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TITLE: Evaluation of Novel Polyunsaturated Fatty Acid Derived Lipid Mediators of Inflammation to
Ameliorate the Deleterious Effects of Blast Overpressure on Eye and Brain Visual Processing
Centers in Rats

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14. ABSTRACT Using an adult rat model of blast wave exposure, we rigorously characterized the cellular and functional damage to the eyes (retinas) and brain visual processing centers, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing the rats in a compressed air driven shock tube and exposing them, in a right side on orientation, once to a 20 psi (260 Hz) blast over pressure wave. Rats were assessed at baseline and then 1, 7, and 14 days post-exposure. By 7 to 14 days out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes for the right side eyes (~ 30% less; n = 15 vs. 14), a trend for impaired ability to visually discern a cue light to earn food rewards (~ 30% less; n = 10 vs. 11), and significant neuronal cell degeneration within the right side retinas and both brain optic tracts (2 and 3-fold more, respectively; n = 11 vs. 11). ERG and histopathology results significantly correlated with each other (r = - 0.7). There also was a strong relationship between the retina and brain optic tract cell damage (r = 0.8). Overall, our findings demonstrate that blast wave exposure leads to loss of vision in rats, likely through retinal cell death followed by anterograde degeneration of brain visual processing centers from lack of signaling input.					
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INTRODUCTION

Blast injury has emerged as arguably the greatest threat to warfighters in current Mideast theaters of operation (Warden, 2006), and is the leading cause of vision loss in military personnel (Cockerham, 2011; Capó-Aponte, 2012). Of blast-related casualties, 43% display closed eye injuries having a 26% incidence of retina damage (e.g., hemorrhaging, tears, and detachments), which is very consistent with a blast wave displacement of fragile ocular tissues (Cockerham, 2011). Although soldiers are issued protective goggles in the field, ocular injuries can still result due to non-compliance of wear, blast wave penetration, or being blown off the face. It is also possible that the brain visual processing centers are being directly affected, since it is well established that blast wave exposure causes traumatic brain injuries (Warden, 2006). Despite the difficult lifelong disability that permanent loss of vision represents, there are only a small number of studies in animals that have attempted to assess blast wave injuries to the visual system (Petras, 1997; Hines-Beard, 2012; Jiang, 2013; Mohan, 2013). All of these prior studies fall short on the soundness of experimental design (e.g., poor blast simulation and/or non-inclusive outcome measures); and only one has looked at potential drug treatments (Jiang, 2013).

First, we purposed to rigorously characterize the cellular, neuronal signaling, behavioral pathology of blast wave injuries to the eyes, specifically the retinas, and brain visual processing centers of adult male rats. These studies are in full progress and have been carried out by subjecting the animals to high fidelity simulated blast over pressure waves (Friedlander waveform), as produced by a compressed air driven shock tube. Eye and brain injuries have been assessed in the rats out to 14 days post-exposure, using well established techniques of electroretinography (ERG; retinal signaling response with a light stimulus), visual discrimination behavioral testing (pressing a lever with a cue light to earn a food reward), and histopathology (H&E and silver stains).

Second, we have purposed to develop new drug therapies that can arrest progression of neuronal cell death in the retina and brain, as result of exposure to blast waves; these studies have not been carried out as yet. Our hypothesis is that novel polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs in healing of neurons critical to visual function after damage from blast wave exposure. Indeed, all of these endogenously produced molecules have been shown to heal ischemic, mechanical, and disease injuries to the retina and brain (Serhan, 2008; Bazan, 2010; Serhan, 2010). Targets for these molecules are G-protein coupled immune-factor receptors (Serhan, 2011). Their basic mode of action is to stop neutrophil migration; block cytokine and eicosanoid release; and recruit monocytes for apoptotic cell removal (Serhan, 2010). Thus, they are excellent drug candidates for our neuronal injury model; and we will screen four - commercially available - sound examples (i.e., lipoxin A₄, protectin DX, resolvin E1, and resolvin D1). Each drug will be intravenously administered to the rats immediately following blast exposure and then once every other day out to 14 days thereafter. Assessment of drug efficacy at alleviating retina and brain neuronal cell damage will be carried out using the previously mentioned outcome measures. Overall, results from our study will provide an important contribution to the understanding and therapy of blast related injuries as translated to man, and thus to the advancement of military as well as civilian medicine.

BODY

I. Submission and Approval of an Animal Protocol for the Project

During the beginning of the first year of the project, we wrote and submitted an animal protocol for the project, which was approved by the WRAIR-IACUC and then USAMRMC-ACURO (07 and 28 January, 2013, respectively). The protocol (WRAIR #: 13-PN-03) is entitled "Evaluation of drugs that are polyunsaturated fatty acid derived inflammation mediators in *Rattus Norvegicus* to ameliorate injuries to the eye and brain visual centers, as induced by exposure to blast over pressure waves". Target of the protocol for clinical significance is to show in a rat model of blast wave exposure the efficacy of at least one of four drugs - that are known polyunsaturated fatty acid derived pro-resolving mediators of inflammation (i.e., lipoxin A₄, protectin DX, resolvin D1, and resolvin E1) - to protect the eye (retina) and brain visual processing centers against neuronal cell damage. These drugs will be individually given to the rats by intravenous injection following blast wave exposure and the retina and brain injury outcomes will be measured using electroretinogram (ERG) recordings, visual discrimination testing, and histopathology.

Based on many previous blast injury studies done on rats by our group, it was estimated that 12 animals per treatment group would be required to show statistically significant differences versus sham and/or blasted rats. During review of the protocol, the WRAIR-IACUC strongly felt that our previously obtained data was not appropriate for basing the group sizes chosen for this project (n = 12); and this was primarily due to the dissimilar experimental conditions and outcome measures that are being used here. It was suggested by the WRAIR-IACUC that we carry out experiments on 6 - 12 shams and blasted rats and use the

resulting means and variances from each outcome measure to perform power calculations to find the appropriate group sizes for the rest of the study. We were to eventually report these results back to the WRAIR-IACUC, which would then adjust the total animals allowed on the protocol, if necessary. We did not do these preliminary power calculations and instead proceeded to do 14 shams and 15 blasted animals, since early on it became obvious that at least 12 animals were needed to pull out significant differences between groups, which we have achieved for the ERG and histopathology outcome measures. Consistent with this, the visual discrimination test has not yet found significant differences with groups of 11 sham and 10 blasted rats. We will, however, perform power calculations using our accumulated data before the start of the drug testing phase of the project to help streamline our animal usage or to justify requests for additional rats for the study.

Finally, there were no official modifications to the protocol put in place or pending during the first year. We do eventually intend to submit a revised SOW and justification regarding a request for an additional 32 rats beyond the 96 originally purposed in the grant. This is mostly to cover larger than anticipated group sizes for the visual discrimination test. Currently, the IACUC / ACURO approved protocol allows for 120 rats, including spares; therefore, we will have to also submit a protocol amendment for adding 8 rats. I will also submit a request to the USAMRMC / TATRC for supplemental funds to support the additional animal costs as outlined in a revised SOW. A total of 40 rats were requested / used during the first year. We put in our last request for animals (8 - total) on 24 September, 2013, to initiate the experimental drug testing phase of our study. These rats will arrive at our facility in the 2nd year period on 16 October 2013. Thus, the remaining animal balance on the protocol is a total of 80 rats.

II. Induction of Eye and Brain Injuries using Exposure to Blast Waves

Adult male Sprague Dawley rats (2 months-old) are placed under brief anesthesia using isoflurane gas. Anesthetized animals are put in a prone transverse position inside a nylon mesh sling that is secured to a metal frame sled. Rats are positioned with right side of the body perpendicular and opposite to the sled, and hence right eye facing the oncoming blast wave during exposure. In this manner, the left eye serves as a control, expected to incur less severe injuries or none. The rat-loaded sled is inserted down the barrel of a compressed air driven shock tube to a preset position in its forward expansion chamber. The unawake animal is then exposed to a single air driven blast wave with a main harmonic frequency at 260 Hz and a peak over pressure of 138 kPa (20 psi). The blast wave is generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness, to deliver 20 psi of air to the rat's position, as clamped between the rear compression and forward expansion chambers. The blast wave travels by the rat with a Mach 1.34 shock front speed, 62 μ sec rise time, 6 msec duration, 281 mph (126 m/s) wind speed, and an acceleration g-force of > 1000 g. Blasted rats are immediately removed from the shock tube and monitored on a thermal blanket during recovery. Animals exhibiting stable respiration and awakening signs are returned to their housing cages. Shams are subjected to isoflurane anesthesia and recovery procedures as described above, but not to blast waves. Sham and blasted rats are then used for ERG or visual discrimination behavioral testing, as to be described below.

Over the course of the first year period, we successfully exposed a total of 16 rats to blast waves, along with 16 aged matched shams. This blast wave exposure procedure has been well established in our laboratory for producing mild to moderate traumatic brain injuries in rats, usually with accompanying retina damage. One major concern that we do have with this technique, however, is the potential for Mylar membrane fragments or animal holder netting to strike the rat's eyes and cause extraneous injuries during the blast wave generation. Indeed, some of the contusion marks we have observed on the rat's eyes post-blast are high up on the sclera near the corneal base, suggestive of netting or Mylar fragment strikes. Consistent with this, we often find Mylar dust at the position of the rat in the expansion chamber following blast; and occasionally microscope fragments of Mylar are embedded in the animal's cornea. We have considered putting protective gauze patches over the rat's eyes, but this could lead to dampening or distortion of the blast wave upon impact. Another major concern we have with the procedure is many rats come out of the shock tube exhibiting severe signs of apnea. If breathing has ceased, we immediately perform CRP (chest massage and oxygenation) on the animal until vital signs are restored. We did not have any rats die during blasting or within 24 hours afterwards, yielding an excellent survival rate. This procedure, however, could still have produced transient ischemia in rats afflicted with apnea. It is known that the retina and brain are hypersensitive to lack of oxygen; and thus ischemic conditions could exacerbate any neuronal cell damage due to blast alone. We could resort to mechanical ventilation of all rats for a short time period immediately post-blast, if respiratory problems continue to be an issue.

III. Electroretinogram (ERG) Recordings of Sham and Blasted Rats

Materials and Methods

Rats are adapted in full darkness for at least 5 hours, prior to being ERG tested. The dark adaptation is done to prime the retina light signaling responses and reduce retinal neuron background noise. Rats are then placed under anesthesia using isoflurane gas and pupils dilated using drops of tropicamide and phenylephrine (cholinergic antagonist and α -adrenergic agonist, respectively). The rat's eyes are also numbed with drops of propracaine. The animal, while maintained on gas anesthesia through a nose cone, is placed on a thermal blanket and a ground electrode fixed to the tail and reference electrodes to both cheeks, using short sub-dermal pins. Recording electrodes are attached to each cornea by placing the fine silver wire leads under contact lens affixed with methylcellulose solution. The rat is laid prone with its face fully inserted into the light stimulus dome of a Handheld Multispecies electroretinogram unit (HMs-ERG; Ocuscience, Inc.). The eyes are then given a scotopic ERG exam (i.e., dark adapted response), using a light stimulus program that exposes the eyes to a series of white light flashes of six increasing intensities (i.e., 100, 300, 1000, 3000, 10,000, and 25,000 mcd.s/m²), with each repeated 1 - 4 times (averaged) at an interval of 10 sec and a duration of 5 msec, and having a ramp spacing of 30 - 60 sec. This program was recommended to us by the manufacturer, for obtaining reliable ERG results on rats (i.e., a broad-range flash response curve). ERG responses arising from each eye are recorded simultaneously by computer and the peak voltage amplitudes of the underlying a- and b-wave forms and their implicit times (i.e., delay from zero to peak) are derived to judge the functional status of the retina photoreceptors and bipolar / amacrine neurons, respectively. After the ERG exam, to protect their dilated eyes from bright light damage, the rats are kept in darkness for at least several hours until they are recovered from anesthesia and pupil constriction reflex is restored; and then they are returned to their normal housing cages under standard lighting conditions. Rats are given an ERG exam at 1 day prior to blast over pressure wave exposure to establish their baseline light stimulus responses, and then retested once at 1, 7, and 14 days afterwards.

Results and Specific Conclusions

Over the course of the first year of the project, we successfully carried out scotopic ERG recordings on a total of 14 sham and 15 blasted rats at 1 day prior to injury (baseline) and at 1, 7, and 14 days thereafter. We did have some problems with the isoflurane anesthesia used to sedate the rats during the procedure and had 3 additional rats die (2 shams and 1 blasted) likely due to respiratory or cardiac failure. While this is only a 9% death rate, we have adjusted the isoflurane delivery system to provide better oxygen flow, and more carefully monitor the rat's breathing and reset the isoflurane to oxygen ratio accordingly. In order to make the ERG data easier to present, only the peak amplitudes and implicit times for the resulting a- and b-wave responses at the light flash intensity of 3000 mcd.s/m² are plotted out versus time post-blast. This flash intensity is recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) as an optimal light stimulus for doing ERG recordings in research animals and humans.

Shown in Figure 1 (see supporting data section) are the eyes and ERG traces for a blasted alive-rat at 7 days out. The right eye shows marked corneal scarring and little light signaling response. Necropsy of this animal at 14 days post-blast showed the right eye to be atrophied with severe retinal degeneration. The left eye's exterior shows some redness and ERG waveform's amplitudes and implicit times appear normal, except the b-wave has oscillatory spikes that are suggestive of neuron misfiring; but at 14 days post-blast the external and retinal pathology of this eye was normal. Next in Figure 1 are shown the bar graphed ERG amplitudes and implicit times for right and left eyes of shams versus blasted animals (mean \pm SD; n = 14 and 15) at baseline and then 1, 7, and 14 days following exposure. Baseline ERG is recorded at 1 day prior to blasting. Light flash stimulus used here is 3000 cd.s/m².

We found that the right eyes of blasted rats had statistically significant decreases in a- and b-wave amplitudes at 7 days post-exposure when compared to its baseline and sham values (31, 30, 30, and 33%; p = 0.006, 0.006, 0.006, and 0.002, respectively), and at 14 days post-exposure versus only its baseline (24 and 22%; p = 0.03 and 0.05, respectively); but no differences were seen at 1 day out. These findings strongly indicate there is substantial blast induced retinal injury on the right side, which faces the shock wave. In contrast, the left eyes had no pronounced differences in ERG responses; where there was only a minor significant decrease in a-wave amplitude at 14 days post-blast (13%; p = 0.03). This indicates negligible functional impairment is occurring to the left eye following blast, which is consistent with it being opposite to the oncoming shock wave. While ERG amplitudes were decreased in the blasted rats, there were no significant differences for both eyes detected for a- and b-wave implicit times at any day post-exposure, implying the retinal deficits are likely due to neuronal cell death (e.g., photoreceptor losses), as opposed to impairment of signaling rate in living cells. The modest differences detected here between sham and blasted rats are not due in part to high same animal variability in the retina light responses from resting state, since we found that dark adapting the rats overnight (16 hours) versus 5 hours prior to

the ERG exam did not enhance or further stabilize the amplitudes or implicit time for the resulting waveforms (n =6; data not shown). Also, we found that back to back ERG reruns done on some animals to verify results gave nearly identical readings. There were some concerns that the ERG testing itself could lead to disturbances in retina function due to factors such as repetitive exposure to the light flash stimulus. Shams, however, were not found to significantly decline in ERG amplitudes or implicit from baseline out to 7 and 14 days of testing; neither was there an apparent trend at 7 days for this phenomenon. It should still be taken into consideration that the ERG procedure may exacerbate any blast wave injuries to the eye and thus should be kept to a minimum as much as possible. We may consider exposing the rats to just a single light flash intensity (i.e., 3000 mcd.s/m²) or dropping the 1 day post-blast measurement.

IV. Visual Discrimination Testing of Sham and Blasted Rats

Materials and Methods

Animals are placed inside visual discrimination conditioning boxes (Med Associates, Inc.), consisting of a standard housing cage that is equipped with a response lever, a cue light mounted above the lever, a water bottle, and a recessed food trough connected to a dispenser capable of discharging small pellets of standard rodent chow. The boxes also have an internal house light, which is continually left on during the animal's entire stay inside. Training the animals for the vision test consists of a sequence of three individual program phases presented to the rats over four sessions. For the initial training session, rats are placed in the conditioning boxes for a 12 hour overnight period. This session consists of two phases. The first phase simply cycles the cue light on and off in conjunction with extending the response lever out and in. The aim is to draw the rat's attention to the lever and get it to press the lever while out and the cue light is on. During each trial, the cue light and lever stay active for 30 sec. Pressing the lever during this time rewards the animal with a food pellet treat. If the lever is not pressed during the active period, a timeout occurs. The cue light goes off and the lever temporarily retracts for a time period (inter-trial interval) randomly chosen between 10 and 30 sec in 5 sec increments. In phase 1, however, a free food pellet is issued every 20 min to help stimulate the rat. After 100 correct lever presses in phase 1, the program moves to the second phase, where the lever is always left in the extended position while the light cycles on and off and free food pellets are not issued. Again, the goal here is to achieve 100 correct lever presses only while the cue light is on.

The second training session is also a 12 hour overnight session that begins with phase 2 (or a phase 1 repeat, if necessary). The active cue light / lever period here is reduced from 30 to 15 sec, and again no free food pellets are given. After 100 correct trials, the program moves onto a phase 3 in which a punishment is introduced when the rat incorrectly presses the lever while the cue light is off. During this phase and all later testing, the punishment consists of turning off the boxes' house light and retracting the lever for 15 sec. The animal then goes on to training sessions 3 and 4 that utilize a 2 hour time period each with no limit on earned food pellets (correct responses), when running phase 3. These two sessions are meant to reinforce the concept of depressing the lever while the cue light is on and reduce the amount of guessing (i.e., depressing the lever when the cue light is off). For these sessions, the active cue light / lever period is reduced to 8 sec. A correct response accuracy of at least 60% at the end of the training (session 4) is our absolute criterion for the animals to move forward into actual visual capacity testing following blast exposure. We do not have a clear explanation for failure of some rats to learn the test, other than they may be simply uninterested in the food pellet rewards or overly anxious of the test environment. While we do not continue on with visual discrimination testing of non-performing rats, they are retained as sham or blasted animals and then subjected to ERG recordings and histopathology, as scheduled in the project.

Finally, baseline visual discrimination tests are performed for successfully trained rats on the day prior to and in the morning directly before blast wave exposure (days 8 and 9, respectively). In these tests, the program runs through a scrambled order of cue light intensity levels with random inter-trial intervals as described above until 117 trials (9 at each of 13 cue light levels) have been completed. For our scale, each cue light level is roughly an 8% reduction in intensity of the previous one, ranging from maximum brightness down to near zero output. At 2, 5, 8, 12, and 14 days following blast wave exposure, the rats are retested against the randomized light intensities for 2 hours. Number of correct responses / food pellets earned (i.e., pressed the lever only when the cue light was on) will be used to determine the animal's visual capacity threshold. We have tried to design the task to be an acuity test as opposed to purely a memory test.

Results and Specific Conclusions

Over the course of the first year period, we ran a total of 32 rats through visual discrimination testing; however, only 21 of those were fully carried on for 14 days past the initial training phase due to deaths during the ERG exam (9% death rate) and inability to master the test (31% failure rate). For the most part, rats that failed to adequately master the test appeared

to be uninterested in obtaining the food rewards, even though we limited their total food intake (3 - 4 pellets) the night before the test as a motivational tool. Rats that passed the training period were grouped as 11 sham and 10 blasted animals. Shown in Figure 2 (see supporting data section) are the bar graphed visual discrimination responses (i.e., lever presses with a cue light to earn a food reward) for sham versus blasted rats (means \pm SD; $n = 11$ and 10 , respectively) at baseline and then 2, 5, 7, 12, and 14 days following blast wave exposure. Baseline responses were those recorded in the morning directly before blasting. While the two groups did not significantly differ at any time point for total, correct, and incorrect lever responses, there was an apparent trend over time, peaking at 7 days post-injury, for the blasted rats to have a higher number of total and incorrect responses compared to shams (1.3 and 1.6 - fold; $p = 0.24$ and 0.20 , respectively). We did try relaxing the stringency for the statistics from a two to one tailed t-test, but this still did not achieve significance ($p = 0.12$ and 0.10 , respectively).

During the experimental drug testing phase of the project, we will be adding 12 more each of sham and blasted rats, as drug controls, to the current results; and thus, will greatly improve our chances to pull out a significant difference between these two groups, which is currently headed in the right direction. The trend in differences as it stands right now, however, leads one to reasonably speculate that the blasted rats are perhaps simply "guessing" more during the test to score a similar tally of correct response (e.g., frequently hitting the lever at random). Indeed, the blasted rats appear to have a lower ratio of correct "hits" out of total responses compared to shams (0.36 and 0.49, respectively). We will eventually look into whether the blasted rats required a greater light intensity at which they were capable of obtaining correct responses (i.e., acuity threshold).

Overall, our visual discrimination findings are consistent with the degree and timing of those we found for the ERG recordings, i.e., peak deficits (~30%) at 7 days post-blast with substantial recovery signs at 14 days. We also carried out Pearson's correlation analysis between the ERG amplitudes (a- and b-wave; right eye) and visual discrimination incorrect lever responses both at 7 days ($n = 10$, each; graph not shown) and found there was not a significant relationship between the two ($r = -0.38$ and -0.34 ; $p = 0.28$ and 0.33 , respectively). This finding is consistent with the lack of significant differences for the visual discrimination test, but may also indicate deficits during this test are due to factors besides retina damage, such as unrelated brain dysfunction (e.g., memory and learning deficits). Blast injured rats could also be using other senses to work around the test (e.g., hearing cue light relay switches activate), thus damping any differences due to vision loss.

V. Histopathology of Eyes and Brains from Shams and Blasted Rats

Materials and Methods

At 14 days post-blast wave exposure, after a final visual discrimination test and ERG exam, rats are euthanized for tissue collection. Animals are anesthetized with isoflurane and then perfused transcardially with saline, resulting in euthanasia by blood exsanguination, followed by 4% paraformaldehyde saturated with picric acid. Prior to saline perfusion, a blood sample is taken by cardiac puncture. Liver lobe is also collected and quick frozen on dry ice. Blood is later spun to obtain the plasma fraction. Plasma and liver are stored frozen at -80°C for use by other investigators in our lab. After perfusion, whole brain and eyes are removed; and observational notes and pictures are taken to record the gross external pathology.

Tissues are then subjected to further processing over several days with other fixative reagents. Brains are washed in sucrose solution. Eyes are post-fixed, to harden the globes, with isopropanol, trichloroacetic acid, zinc chloride, and ethanol. Fixed eyes and brains are sent out to FD NeuroTechnologies, Inc. (Ellicott City, MD) to be made under a contract agreement into slides containing cross sections stained with hematoxylin and eosin / H&E (eyes and brain) and silver (brain only), to hunt for signs of neuronal apoptosis as indicated by cell morphology disturbances and axonal fiber tract degeneration, respectively. Eyes are cut in a single horizontal section ($5\text{ }\mu\text{m}$) through the pupil's central axis. Brains are cut in 11 evenly-spaced vertical sections ($30\text{ }\mu\text{m}$) through the cerebrum, to cover all underlying visual processing centers. Prepared slides are examined under an axial light microscope equipped with an image capture camera and a computer having image processing software. For the brains, neurons in visual processing centers known to be effected by blast injury are assessed on the slides (e.g., optic tract, optic chiasm, superior colliculus, geniculate nucleus, and occipital cortex). For the eyes, distinct neuronal layers making up the retina are examined (e.g., ganglion, bipolar / amacrine, and photoreceptor cells). Brain and retina injury status is quantified using gross observations (e.g., bleeding, tearing, and swelling), cell layer thicknesses, cell body counts, and staining densities. Likewise, for external injuries to the eye globes, degree of contusions, inflammation (e.g., corneal redness), and lens cloudiness are noted. Injuries are assigned relative damage scores, using a rank scale of 1 - 6 (e.g., none, slight, mild, moderate, severe, and catastrophic), as judged by consensus of one to two "blinded" reviewers (lab technicians) and one "un-blinded" moderator (senior scientist) who advises on regions of interest (e.g., artifacts versus injury) and settles score split decisions.

Results and Specific Conclusions

Over the course of the first year period, we submitted eyes (right and left pairs) and brains from 14 shams and 15 blasted rats, collected at 14 days post-injury, for histopathology processing by an outside contract company (FD NueroTechnologies, Inc.). The eyes and brains are made into H&E (eye and brain) and silver (brain only) stained microscope slides. To date we have received back eye and brain slides for 11 shams and 11 blasted rats and put them through the relative damage scoring processes. We first did comparisons between all of the sham and blasted rats done for external eye globe injuries at 14 days post-blast. Shown in Figure 3 (see supporting data section) are representative images for eye globes from sham and blasted rats. Both eyes of the blasted animal display red contusion marks to the sclera. Also shown in Figure 3 are the bar graphs for relative damage scores (rank scale shown; see inset) of the external globe injuries of sham and blasted rats (means \pm SD; $n = 14$ and 15 , respectively); where we found significantly more damage (1.6 - fold; $p = 0.04$) was present on the right eyes of blasted rats, but not on the left eyes. The closed eye injury incidence for the right side was 67%, as based on these scores. Pearson's correlation analysis (graph not shown), however, found the relative damage scores to not have a significant relationship with those later determined (see below) for the right side retinas ($n = 11$, each; $r = 0.32$, $p = 0.34$). This indicates that external globe injuries are mostly confined to the eye's surface (i.e., sclera) and are not a suitable biomarker of retinal integrity.

We next did comparative assessments between sham and blasted rats ($n = 11$, each) for neuronal cell damage to the retina and brain visual processing centers at 14 days post-blast. Shown in Figure 4 (see supporting data section) are representative microscope images for the right and left side retinas and brain optic tract and superior colliculus regions (20x and 4x magnifications, respectively) of sham and blasted rats. Retinas are the dark purple ribbon "like" structure with distinct neuronal cell body layer divisions. Brain optic tracts are the dark brown oval "like" structure sandwiched between two lobes; whereas, the superior colliculi are the muffin "like" structures that sit atop the mid brain. The retinas and brain regions of the sham are free of obvious cellular perturbations. The right retina of the blasted rat, however, shows marked reorganization and degeneration of the photoreceptor and bipolar / amacrine cell layers. Correspondingly, the right and left optic tracts and superior colliculi of the same animal show black staining consistent with axonal fiber tract degeneration. The left optic tract and superior colliculus shown here are more intensely stained than the right side, which may be explained by the optic nerve fiber bundles from the retinas largely switching hemispheres after the optic chiasm. This implies that much of the brain axonal degeneration is coming from loss of afferent signaling input from the right retina (i.e., anterograde degeneration). However, the presence of marked axonal degeneration on both sides of the brain could also indicate that some of the neuronal damage is from the blast wave displacing these visual processing regions alone.

Also shown below in Figure 4 are the bar graphs for the relative damage scores (rank scales shown; see insets) of the retina and brain optic tracts for shams and blasted rats (means \pm SD; $n = 11$, each); where we found significantly more neuronal cell damage (2.3 - fold; $p = 0.003$) to be present in the right retinas, but not left side, and in the right and left optic tracts to the same degree (3.4 and 3.5 - fold; $p = 0.00004$ and 0.00004 , respectively). The right retina and left and right brain optic tract injury incidence was 82, 100, and 91%, respectively, as based on these scores. We are still in the process of scoring the superior colliculus and other interconnected brain visual processing centers. We also plan to back up this data with cell body counts and layer thickness for the retina and silver staining optical densities for the brain visual processing centers. Limitations to these additional measures, however, are defining the specific regions of interest to assess for the retinas and finding a consistent background to subtract from the brain optical densities. Overall, the retina and brain relative damage scores found here strongly support our current contention that blast wave exposure leads to a double component injury to the visual system, which is likely a combination of direct retinal cell layer damage, anterograde degeneration of brain visual pathway nerve fiber bundles (e.g., retina to optic nerves to optic tracts), and direct axonal shearing of brain regions. It is also possible for directly damaged brain nerve fiber bundles to stimulate degeneration backwards into the retina (i.e., retrograde degeneration) through loss of efferent signaling input.

As a post-hoc comparison, we have carried out Pearson's correlation analysis between the retina and brain histopathology results and those of the ERG (amplitudes) and visual discrimination test (incorrect responses). For simplicity of drawing conclusions, for the most part, only the results from the right eyes or retinas and their signal input corresponding left optic tracts were used for these comparisons. As shown in the scatter plots of Figure 5 (see supporting data section), we compared the right retina and left optic tract relative damage scores ($n = 11$, each) and found there was a highly significant positive relationship between the two ($r = 0.81$; $p = 0.003$), despite the brain's left side being contra-lateral to the blast wave impact. In contrast, relative damage scores for the right retinas and right optic tracts did not correlate (graph not shown; $r = 0.12$; $p = 0.73$). This greatly supports our histopathological evidence that blast wave injury to the retina is leading to anterograde axonal degeneration of the opposing brain visual processing centers.

As also shown in Figure 5, we then compared the right eye ERG amplitudes (a- and b-wave) at 7 days post-blast to the right retina and left optic tract relative damage scores ($n = 11$, each) and found they all had significant negative relationships between each other ($r = -0.76$, $p = 0.006$; $r = -0.72$, $p = 0.01$; $r = -0.77$, $p = 0.006$; and $r = -0.78$, $p = 0.005$, respectively). This implies that the ERG deficits we saw in the blasted rats are a direct measure of retina signaling function; and helps eliminate other potential causes, such as cornea or lens damage. However, when comparisons were made using the ERG data at 14 days post-blast, we did not find correlations with retina and optic tract relative damage scores (graphs not shown; $r = -0.25$, $p = 0.45$; $r = -0.30$, $p = 0.36$; $r = -0.24$, $p = 0.48$; and $r = -0.32$, $p = 0.33$, respectively). This finding is not surprising, since the ERG deficits showed signs of substantial recovery at 14 days post-blast. Finally, we compared the visual discrimination test incorrect responses at 7 days post-blast and right retina and left optic tract relative damage scores ($n = 7$, each) and found none of these correlated with each other (graphs not shown; $r = 0.48$, $p = 0.28$; and $r = 0.30$, $p = 0.52$, respectively). Again, this finding is not surprising, since none of the visual discrimination test results were significantly different between shams and blasted rats; and the ERG amplitudes did not correlate with this data either.

VI. Initiation of Phase II; Testing of Experimental Drugs to Alleviate Neuronal Cell Death Post-Blast

We now consider phase I of the project - "Characterization of blast injuries to the eyes of rats" - to be satisfactorily completed. While significant differences were not attained on the visual discrimination test between shams and blasted animals, we did find by ERG and histopathology significant retina and brain visual processing center deficits in the rats by 14 days after blast wave exposure. This is an acceptable injury model to begin the second phase of the study with, in which we will test the efficacy of 4 experimental drugs that are known polyunsaturated fatty acid derived lipid mediators of inflammation to alleviate the neuronal cell death in the retina and brain processing centers following blast wave exposure. During the last month of the first year period, we initiated critical steps needed to begin these studies. The four experimental drugs, lipoxin A4, neuroprotectin DX, resolvin D1, and resolvin E1, were ordered from the sole source provider, Cayman Chemical Company. The compound resolvin E1 will be provided to us through a custom synthesis agreement with Cayman Chemicals, while the other three drugs are kept in stock at the company. Due to the difficulty of the synthesis steps and the large amount requested, the estimated lead time for receiving the resolvin E1 will be 4 - 5 months from order date (i.e., January - February, 2014). Thus, we have decided to test the other three compounds first in the animals and save testing of the resolvin E1 for last.

The drugs lipoxin A4, neuroprotectin DX, and resolvin D1 have now arrived at our laboratory, but initially with stamped expiration times of 4 - 8 months from purchase date. Unfortunately, this would provide us with little flexibility on getting the animal testing done on each drug throughout the second year period. We were also concerned that these compounds were sold to us out of old lots that maybe already partially degraded. Thus, we requested a new quality control (re-QC) assessments be done on each drug and certificates of analysis and accompanying purity chromatograms be sent to us. Cayman Chemicals promptly preformed the analysis on each lot and gave us the re-QC reports. Each drug is now certified to be $\geq 95\%$ pure at a concentration of ~ 100 $\mu\text{g/ml}$; and with a new expiration date of mid-September 2014, if stored in the ethanol shipping solvent at -80°C . We will show the full results of the re-QC analysis (HPLC and LC/MS-MS chromatograms) in our first report for the second year period (i.e., 5th quarterly report).

Finally, all of the experimental drugs will be individually administered to the animals, by intravenous injection into the lateral tail vein (25 $\mu\text{g/kg}$; single bolus dose), within 15 min following blast wave exposure; and thereafter given every other day out to 14 days, for a total of 7 doses. The booster shots are necessary to maintain their plasma circulating and tissue uptake / incorporation levels as well as any inflammation knock down status in the blast-injured retina and brain. A major concern we have is that this will be a difficult injection route to perform on rats, because of the small diameter of the tail vein and low visibility under the skin. Thus, we have requested that the WRAIR Veterinary Medicine staff provide us with hands on refresher training for tail vein injections prior to the actual experiments that will start during the second year period on the 30 October, 2013. They are also willing to assist us during regular work days with the injections. Due to the lack of availability of our laboratory personnel and Veterinary Medicine assistance on the weekends, however, we may consider skipping the drug injections during those days (i.e., Saturday and Sunday) and just give the rats their usual booster doses on the proceeding Friday and following Monday mornings.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Wrote an animal protocol for the project that was fully approved by the WRAIR-IACUC and USAMRMC-ACURO. This was assigned as WRAIR protocol #: 13-PN-03 and entitled: "Evaluation of drugs that are polyunsaturated fatty acid derived inflammation mediators in *Rattus Norvegicus* to ameliorate injuries to the eye and brain visual centers." This part of the project was completed by the 4th month of the award, which according to the milestones of the SOW was 1 month behind schedule.
- 2) Exposed 16 adult male rats (2 months-old) once to blast over pressure waves (20 psi; 260 Hz) in a compressed air driven shock tube, along with 16 age-matched shams. In the course of ERG exams (see below), two shams and one blasted animal died, due to isoflurane anesthesia overdosing. The surviving 14 shams and 15 blasted rats were put through ERG exams, visual discrimination testing, and histopathology (eye and brain) as described below; except 3 shams and 5 blasted rats were dropped from visual discrimination test due to training problems. This part of the project was completed by the 8th month of the award, which according to the milestones of the SOW was 1 month behind schedule.
- 3) Performed electroretinography (ERG) on the right and left eyes of 14 sham and 15 blasted rats at 1 day prior to (baseline) and 1, 7, and 14 post-exposure. This represents a total of 116 ERG exams that were done, which typically took 30 min per animal. We showed that blasted rats compared to their baseline and shams had a 30% decrease in ERG response amplitudes for the right eyes (facing the blast wave) by 7 days post-exposure, demonstrating retinal signaling dysfunction was present after blast-injury. This part of the project was completed by the 9th month of the award, which according to the milestones of the SOW was 2 months behind schedule.
- 4) Performed visual discrimination testing on 11 sham and 10 blasted rats at 1 day prior to (baseline) and 2, 5, 7, 12, and 14 days post-exposure. The rats were also conditioned to do the test over 8 days (5 - times) before a final baseline measurement was taken. There were also an additional 11 rats that were put through at least the conditioning phase, but failed to master the test (8) or prematurely died afterward (3). This represents a total of 286 visual discrimination test trials that were done, which typically took 1 - 2 hours per animal. We demonstrated that blasted rats compared to shams have a trend that peaks at 7 days to make 1.6-fold more incorrect lever responses versus a cue light when trying to earn food rewards (i.e., cue light "guesses"), which is similar in pattern to the ERG results. This suggests that some animals are having difficulty visualizing the cue light, due to retinal or brain visual processing center dysfunction after blast-injury. It could also imply that brain impairments in memory centers (e.g., hippocampus) for the test are occurring. This part of the project was completed by the 9th month of the award, which according to the milestones of the SOW was 2 months behind schedule.
- 5) Performed histopathology assessments on eyes (right and left) and brains collected from 11 sham and 11 blasted rats at 14 days post-exposure. A total of 44 eye and 176 brain microscope slides (2 and 8 each, respectively) have been made for us, and then were scored in house for retina and brain optic tract neuronal cell damage using "blinded" reviewers. Samples from an additional 3 shams and 4 blasted rats are still being processed into slides. We demonstrated that blasted rats compared to shams have 2-fold higher relative damage scores for their right side retinas and 3-fold higher scores for both brain optic tracts. Likewise, the globes of the right eyes had 2-fold greater external damage scores (e.g., contusions), but this did not relate to the degree of retinal injury present. Brain damage was also shown to extend into other visual processing centers (e.g., superior colliculus), but these have not been scored as yet. This part of the project is still ongoing, which according to the original milestones of the SOW is more than 3 months behind schedule.
- 6) In support of our histopathology findings, we demonstrated that there was a tight correlation between the right retina and left brain optic tract relative damage scores, and between both of these and the right eye ERG amplitude deficits. This suggests that the blast wave exposure to the eye is causing retina neuronal cell damage that leads to loss of visual signaling output and subsequent anterograde axonal fiber degeneration in the brain visual processing centers. It is also possible that the brain optic tracts are being directly damaged by the blast wave impact. In contrast, we did not find significant ERG and histopathology correlations with the visual discrimination test results, suggesting there is a minimal impact of the blast injuries on vision dependent psychomotor tasks. This part of the project was an adjunct, but highly informative, post-hoc analysis of the accumulated ERG, visual discrimination, and histopathology data; and thus is not listed in the SOW's milestones.

- 7) Initiated second phase of the project on testing the efficacy of 4 experimental drugs (i.e., polyunsaturated fatty acid derived lipid mediators of inflammation) to alleviate neuronal cell damage in the retina and brain visual processing centers of rats following blast exposure. Procured 3 of the drugs (i.e, lipoxin A4, protectin DX, and resolvin D1) and activated the custom synthesis of the last (i.e., resolvin E1). The first set of 8 rats for the study has been ordered (15 October, 2013 arrival date) and will be divided for testing into 1 sham, 1 blasted control, and 2 blasted ones for each of the 3 drugs. This part of the project was begun in the 12th month of the award, which according to the milestones of the SOW is 3 months behind schedule.

REPORTABLE OUTCOMES

- 1) Using the project's preliminary data, submitted grant application to a DMRDP FY13 CRM-ARATDA sponsored program (on 9 September, 2013) as co-PI under Dr. Long, proposing treatment of blast induced ocular injuries with dietary supplementation of omega-3 polyunsaturated fatty acid. However, after grant board review our application was rejected for funding, as mainly based on issues regarding potential of treatment success.
- 2) Presented a poster on project's findings at the Military Health Sciences Research Symposium (MHSRS), held in Ft. Lauderdale, FL on 12 -15 August, 2013. A copy of the submitted / accepted abstract and poster are attached to this report.
- 3) Gave a slide presentation over viewing the project and its findings to the Geneva Foundation's Scientific Advisory Board during their site visit to the WRAIR on 11 July, 2013.
- 4) Project and its goals for advancing Military Medicine were detailed in an article found in the Geneva Foundation's annual report news letter, released in September, 2013. A copy of the article is attached to this report.
- 5) Developed an animal model, using adult male rats, for blast wave induced injuries to the visual system, which includes the retina and brain visual processing centers. Unlike other similar ocular trauma rodent-models in the literature, this will be the first to utilize high fidelity simulated blast over pressure waves (Friedlander waveform), as generated by a compressed air driven shock tube, to produce the injury. The outcome measures that we used were similar to those by others, but with more refined time points and closer interconnections.

CONCLUSION

We have found in rats that a single exposure to a blast over pressure wave, by 7 days out, leads to retinal signaling dysfunction with neuronal cell damage (e.g., photoreceptor degeneration) as the underlying cause. This in turn, we found this apparently stimulated anterograde degeneration of axonal fiber tracts in the brain visual processing centers (e.g., optic tracts and superior colliculus), due to loss of retinal signaling input. It is known that traumatic injuries to the retina produce anterograde degeneration of axonal fibers feeding into the brain starting at the retina ganglion cell layer, but has been proven reversible with drug interventions ([Thanos, 1991](#); [Avilés-Tigueros, 2003](#)). Some of the brain damage could also be the result of the blast wave directly impacting the nervous tissue. We exposed the rats to a blast wave (20 psi; 260 Hz) that produces mild to moderate traumatic brain injuries in the animals, making it a realistic scenario to what a soldier might experience in the field during attacks from explosive devices ([Warden, 2006](#)). Ocular tissues are extremely fragile, especially the retina, so can they be easily displaced and damaged by a blast wave as it is channeled into the skull's eye sockets. We realize that soldiers are issued protective goggles in the field, but blast induced eye injuries will always be of great risk due to potential non-compliance of wear, blast wave penetration, or being blown off the face. Indeed, the incidence of closed eye injuries in blast exposed soldier is 43%, with 26% of these cases involving serious retina damage and long lasting impairments in vision ([Cockerham, 2011](#); [Capó-Aponte, 2012](#)). Our animal model had a similar externally notable closed eye injury incidence of 67%, but 82% of the rats had internal retinal cell damage. This implies that some retinal injuries maybe overlooked in blast exposed soldiers, especially since we found that 60% of the blasted rat eyes without any apparent external globe injuries still had cellular damage present to their retinas. Additionally, we found that the brain visual processing centers of the blasted rats were damaged to an incidence of at least 81%; which is something to our knowledge that has not been clinically investigated in blast injured soldiers as an underlying pathological component for subsequent problems with vision.

Blast wave injury to the brain visual processing centers of rats has been previously described by our group, using identically made blast waves (Petras, 1997); but no one else has reported this. Others have observed retinal signaling deficits by ERG and cellular damage by histopathology in mouse models of blast wave exposure (Hines-Beard, 2012; Mohan 2013), but the injury is either unrealistically catastrophic (e.g., optic nerve avulsion) or delayed in manifestation (e.g., several months out) due to very poor simulation of the blast waves. For example, two studies fired a high velocity air rifle directly at the mouse's cornea (Hines-Beard, 2012; Jiang 2013) and another put the mouse inside an uncontrolled air expansion blast chamber having an obscure end delivery pressure (Mohan, 2013). In contrast, our model utilizes high fidelity simulated air blast waves (i.e., Friedlander waveform) to induce the injuries; and thus, produces visual system damage of a more realistic degree and time post-exposure to the human condition. Improvement of our blast model in the future may include looking at the visual system injury effects under various conditions that might be encountered by soldiers in the field, such as other orientations to the blast wave (e.g., face on), over a wide range of reasonable pressures (e.g., 10 - 30 psi), repetitive blasts (e.g., double blast at a 1 min interval), or combined primary and secondary insults (e.g., blast followed by weight drop induced skull-concussion). Also, while behavioral impairments in visual acuity tracking reflex (i.e., optokinetics) have been looked at (Hines-Beard, 2012), no one has attempted to translate the retinal injuries into actual loss of performance on vision dependent psychomotor tasks. Indeed, for blasted rats, we saw a 30% decrease in retinal signaling with a 2 and 3-fold more cellular damage in their retinas and brain optic tracts, respectively; however, most of these rats still performed quite well on the visual discrimination task (i.e., pressing a lever with a cue light to earn a food reward); and thus, only non-significant trends in vision related behavioral deficits have been observed so far. This test does have the limitation that it is impossible to be certain that the animal isn't able to work around the test, such as compensating with other senses heightened by loss of sight (e.g., hearing the cue light relay switches go off). There is also a concern that the rat's capacity for memory and learning may play a larger part than considered in the test's outcomes. Improvements of visual behavioral testing in the future may include using a battery of functional tasks, such as novel object recognition, cued maze navigation, and spatial place preference (Crawley, 2007). We could also resort to doing ERGs with light pattern stimulation (pERG), which looks at signals from retinal cells involved specifically in visual acuity processing (i.e., ganglion cells) or visually evoked potentials (VEPs) after similar stimulus, as recorded as electroencephalograms (EEGs) from the brain's occipital cortex (Perlman, 2009).

In the end, our study has provided us an excellent blast wave induced injury model for the next phase of the project, which will be looking at the efficacy of 4 experimental drugs (i.e., polyunsaturated fatty acid derived lipid mediators of inflammation) to alleviate the neuronal cell damage to the retina and brain visual processing centers. Others have shown that drug interventions, using β -adrenergic receptor agonists (i.e., proprietary compounds), can prevent retinal inflammation and apoptosis in rats exposed to blast waves (Jiang, 2013). By out to 14 days, we found there are substantial and easy to quantify retinal signaling deficits and neuronal cell perturbations in the retina and brain, thus even partial reversal by our selected drugs of any of these injury markers would be a positive "hit" with great medical implications. We had indications by ERG exams and visual discrimination testing that the injuries may take up to 7 days to manifest, since impairments in retinal signaling and behavioral responses, respectively, were not observed beforehand. This represents a very wide therapeutic "window" for delivery of our drugs to the treat the visual system injuries, which also would be practical in a human clinical setting. Our histopathology assessments, however, looked at neuronal cell changes only at the chronic time point of 14 days post-exposure and we do not know what earlier cellular events happened, which also is helpful for shaping the therapeutic "window". Improvements of the histopathology in the future would be to look post-blast at acute time points (e.g., 6 and 24 hours) and semi-chronic time points (e.g. 3 and 7 days); where some specialized stains for more acute damage would be TUNEL (DNA damage) and Iba-1 (immune cell infiltration) (Naskar, 2002; Nakazawa, 2006). It would also be interesting to look at chronic time points far beyond 14 days post-blast (e.g., 21 and 28 days), since ERG exams and visual discrimination testing indicated that some recovery of visual function was occurring by then. This, however, may be a transient rebound phase with the injury state gradually worsening thereafter. Progressively slow neurodegenerative diseases, originating from a blast induced insult, are known for the brain, such as chronic traumatic encephalopathy (CTE) (Goldstein, 2012). Histopathology of the retina and brain visual processing centers at far time points post-blast could look for "classic" biomarker proteins of chronic neuro-degeneration, such as p-tau, β -amyloid, and GFAP (Hoshino, 1998; Cao, 2001; Liberto, 2004; Grieciuc, 2011).

Finally, our project is currently limited to neuro-physiological, behavioral, and pathological outcome measures assessed at up to 14 days post-injury and does not allow us to adequately address the biochemical alterations behind any negative changes observed, which could lead to new targets for drug candidate considerations. Characterization of these changes will require future Western blot evaluations of specific proteins in fresh retina and brain tissues collected from animals over a finely divided and extended timeframe post-injury to capture both acute and chronic biochemical effects (e.g., 6 hours, and 1, 3, 7, 14, and 28 days). Proteins examined could be selected from those well recognized as biomarkers of neuroinflammation mediated apoptosis (e.g., COX-2, bFGF, IL-1 β , MCP-1, caspase-3, and TNF- α) and retinal signal transduction (e.g., rhodopsin, Gt- α , and cGMP-PDE) (Cao, 2001; Nakazawa, 2006; Rapoport, 2008; Bailes, 2010; Haung,

2012). Plasma collected from the blasted rats could also be screened for these proteins to see if there is a correlation with the retina and brain levels, as a non-invasive diagnostic tool for judging the presence of neuronal injuries.

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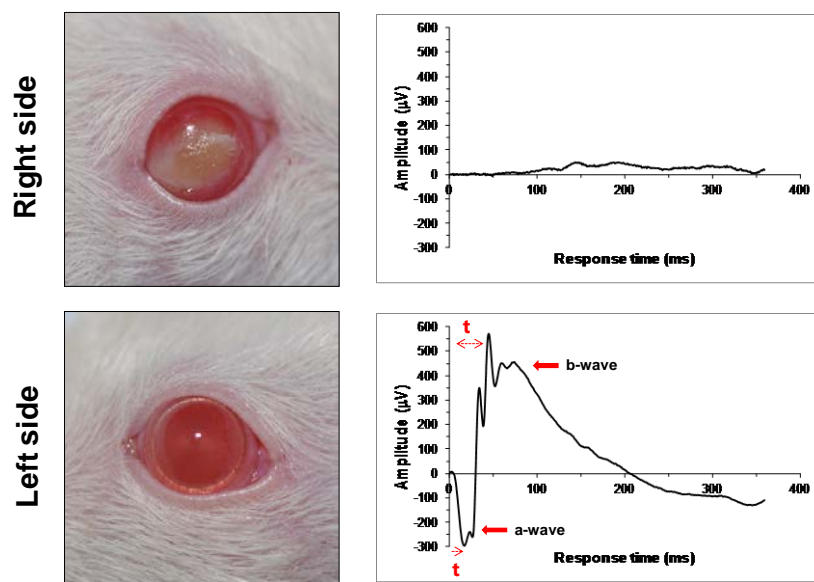
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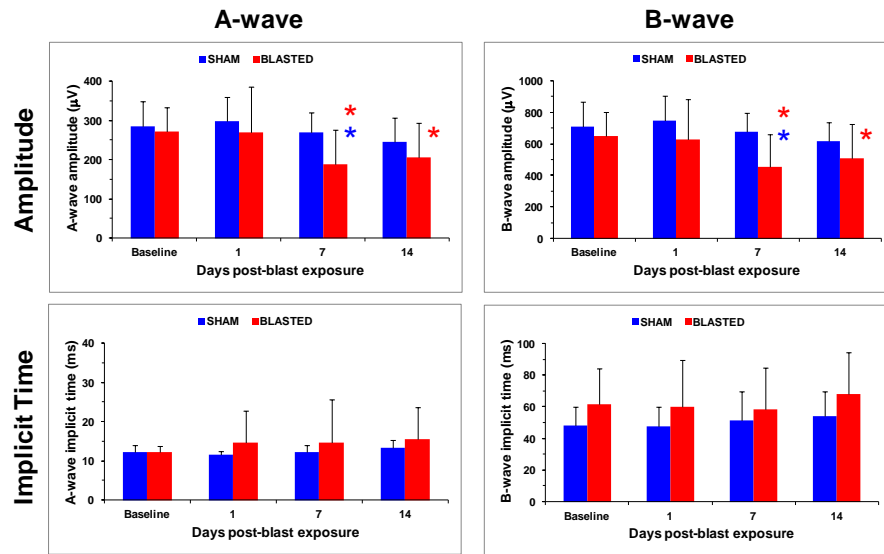
SUPPORTING DATA:

Figure 1: ERG recordings (amplitudes and implicit times) for sham vs. blasted rats

Eyes and ERG Waveforms for a Blasted Rat at 7 days out



Right Eyes



Left Eyes

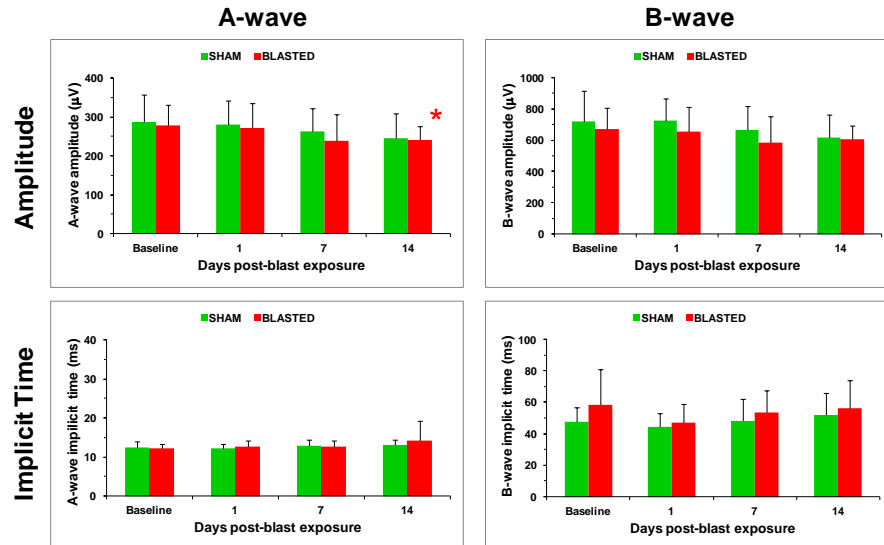


Figure 1. Top panel; eyes and ERG waveforms for a representative blasted rat at 7 days out; the right eye shows marked corneal scarring and little retinal signaling response. Left eye's exterior and ERG trace are relatively normal. The a- and b-wave portions of the ERG trace are indicated by red arrows; t = implicit time. Bottom panels: bar graphs of ERG amplitudes and implicit times for sham versus blasted animals (mean \pm SD; n = 14, 15), as taken at baseline and then 1, 7, and 14 days post-blast exposure. Baseline recordings were all done at 1 day prior to blast. Light flash stimulus used here was 3000 cd.s/m². Panels are separately shown for right and left eyes (blue + red / sham vs. blasted; and green + red / sham vs. blasted, respectively). *p \leq 0.05 vs. blasted baseline; *p \leq 0.05 vs. shams.

Figure 2: Visual discrimination test responses for sham vs. blasted rats

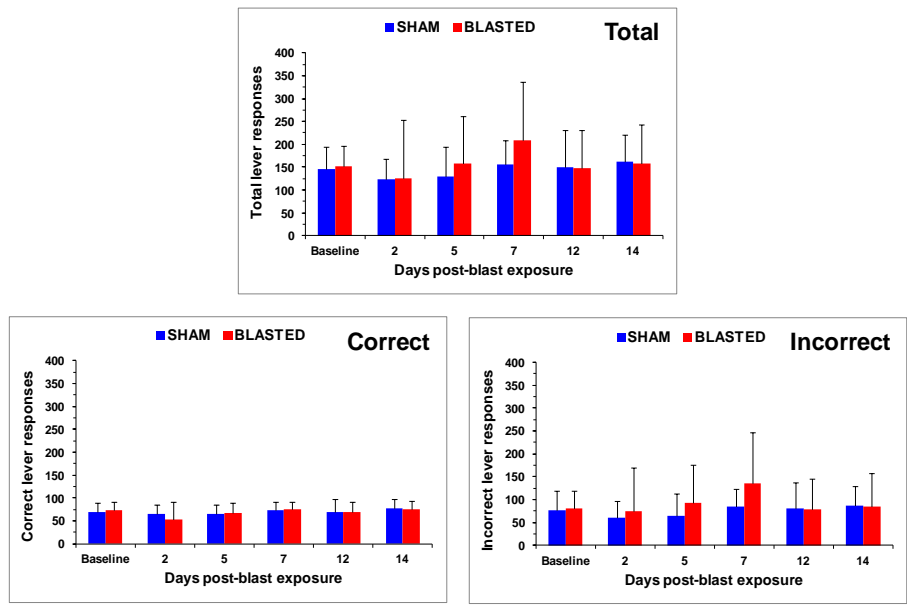
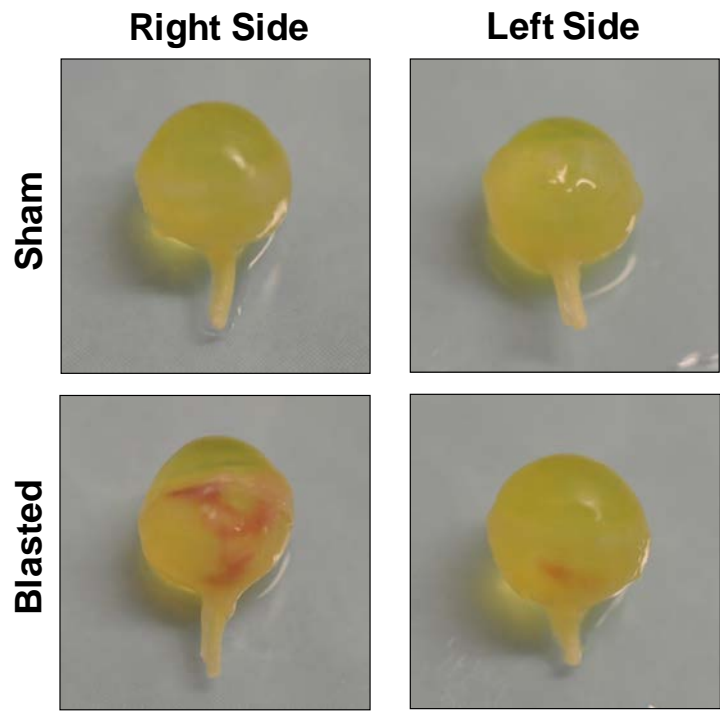


Figure 2. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn a food reward) for shams (blue) versus blasted (red) animals (mean \pm SD; $n = 11$ and 10 , respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast exposure. Baseline was done in the morning before blasting. No significant differences found between groups on any parameter.

Figure 3: Eye globe injuries and their relative damage scores for blasted rats



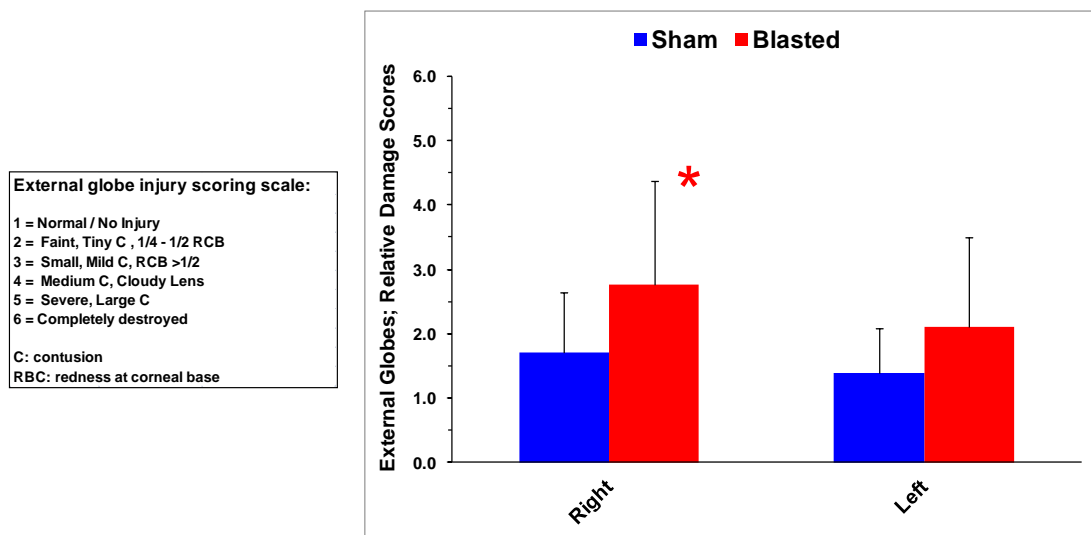
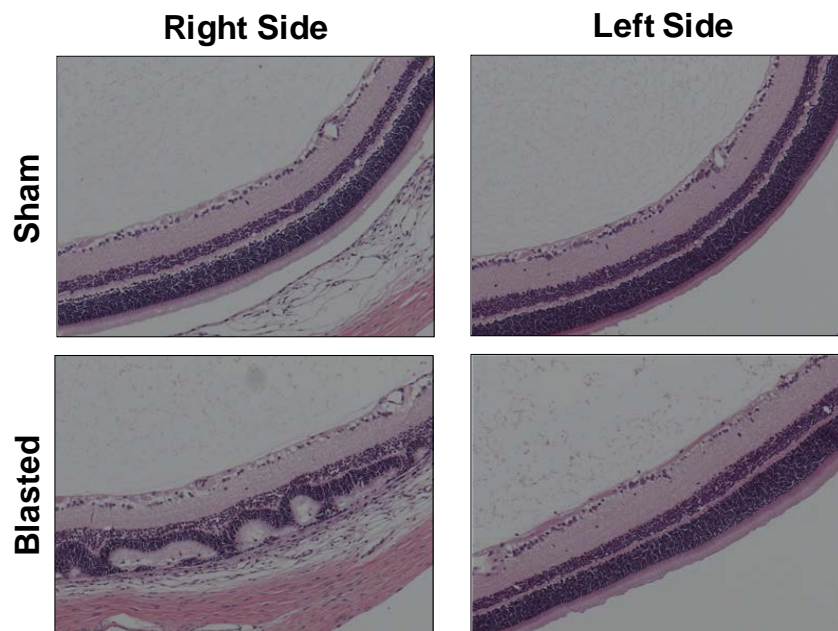


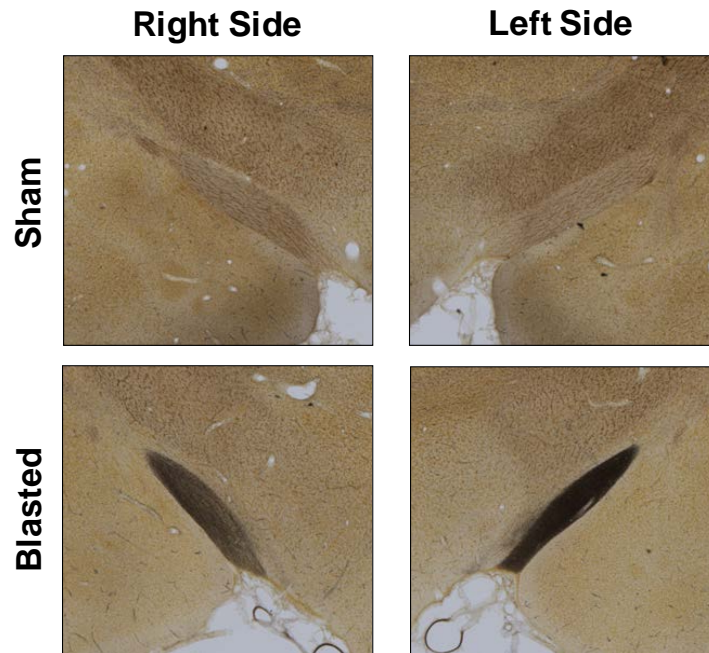
Figure 3. Top panel; representative eye globes (right and left) from sham and blasted rats at 14 days post-exposure. Blasted eye sclera show distinct red contusion marks. Bottom panel; bar graphs for relative damage scores of external globe injuries (right and left) of sham and blasted rats (means \pm SD; n = 14 and 15, respectively). Rank scale (1 - 6) used for scoring is shown in detail in the left inset. *p \leq 0.05 vs. shams.

Figure 4: Retina and brain injuries and their relative damage scores for blasted rats

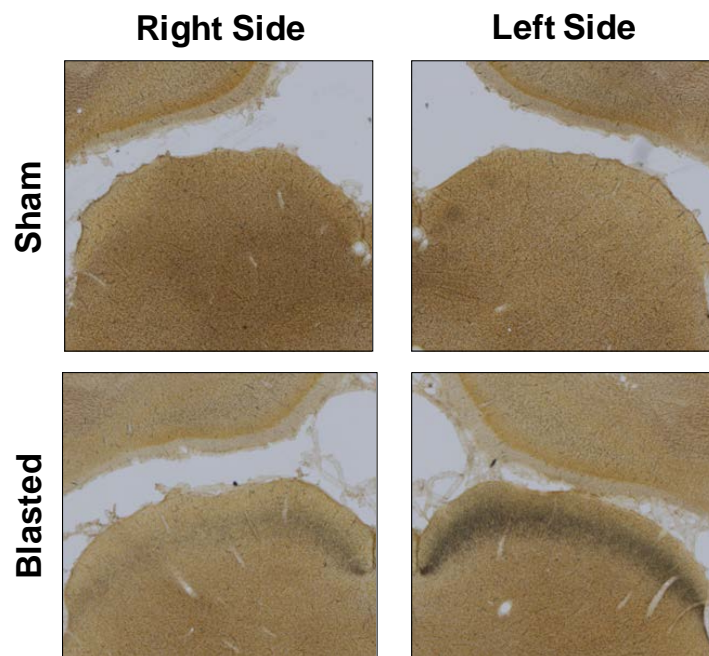
Representative Retinas



Representative Brain Optic Tracts



Representative Brain Superior Colliculi

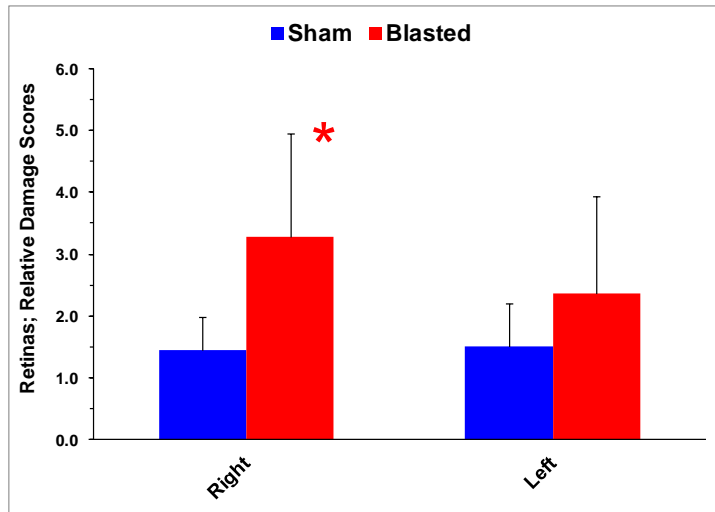


Retina Relative Damage Scores

Retina injury scoring scale:

- 1 = None (0%)
- 2 = Slight (< 20%)
- 3 = Mild (20 - 40%)
- 4 = Moderate (40 - 60%)
- 5 = Severe (60 - 80%)
- 6 = Catastrophic (80 - 100%)

Judged by extent / intensity of retinal swelling and neuronal cell disruption



Brain Optic Tract Relative Damage Scores

Brain optic tract injury scoring scale:

- 1 = None
- 2 = Slight
- 3 = Mild
- 4 = Moderate
- 5 = Severe
- 6 = Catastrophic (pitch black)

Judged by intensity of silver stain coloration

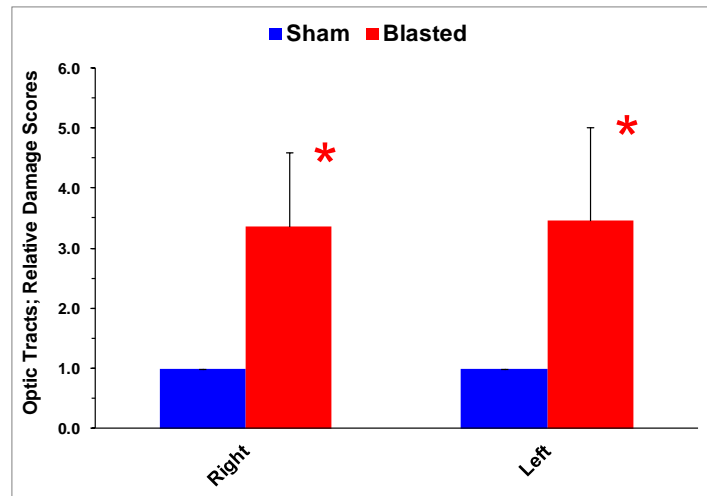
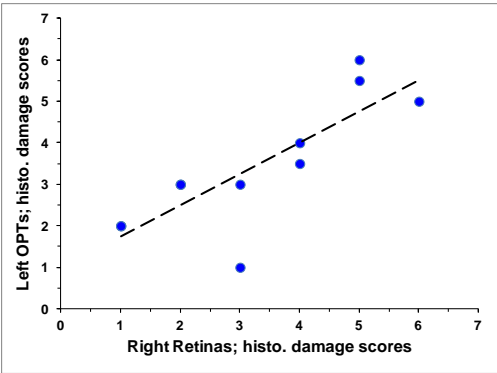


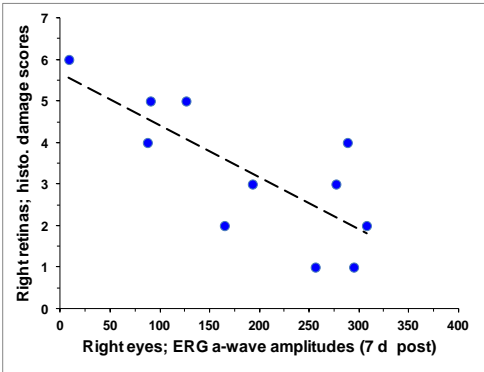
Figure 4. Top 3 panels; representative microscope images for cross sections of retinas and brain optic tracts and superior colliculi from sham and blasted rats at 14 days post-injury, stained with hematoxylin and eosin (H&E) and silver, respectively. Blasted retina sections (20x) show extensive neuronal cell layer degeneration to be present on the right side. Likewise, blasted brain sections (4x) show the presence of marked axonal fiber tract degeneration (black coloration) on both the right and left sides. Bottom 2 panels; bar graphs of relative damage scores for retinas and brain optic tracts of sham and blasted rats (means ± SD; n = 11, each). Rank scales (1 - 6) used for scoring are shown in detail in the left insets. *p ≤ 0.05 vs. shams.

Figure 5: Pearson's Correlation Analysis for ERG versus Histopathology Results

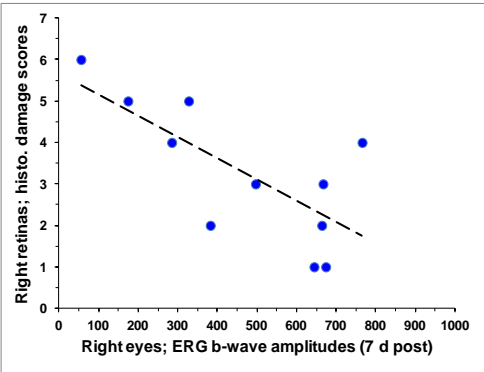
Retina vs. Optic Tract Damage Scores



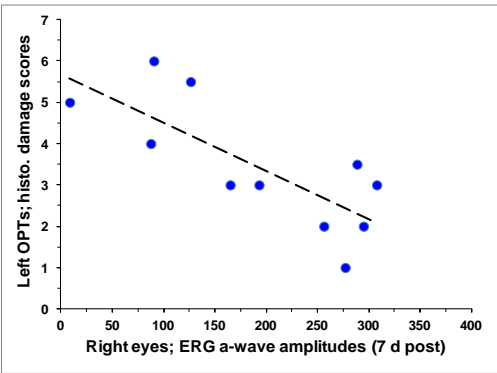
ERG a-wave vs. Retina Damage Scores



ERG b-wave vs. Retina Damage Scores



ERG a-wave vs. OPT Damage Scores



ERG b-wave vs. OPT Damage Scores

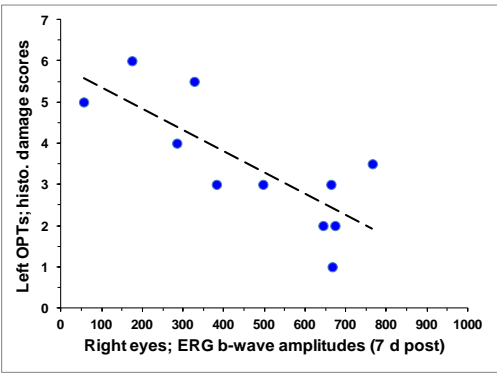


Figure 5. Scatter plots for Pearson's correlation analysis between right retina and left brain optic tract relative damage scores (n = 11, each); right eye ERG amplitudes (a- and b-wave) at 7 days post-blast and right retina relative damage scores (n = 11, each); and right eye ERG amplitudes (a- and b-wave) at 7 days post-blast and left brain optic tract relative

damage scores (n = 11, each). Significant relationships were found for all comparisons (left to right and top to bottom: $r = 0.81$, $p = 0.003$; $r = -0.76$, $p = 0.006$; $r = -0.72$, $p = 0.01$; $r = -0.77$, $p = 0.006$; and $r = -0.78$, $p = 0.005$, respectively).

DISTRIBUTION LIMITATIONS

This report does not contain any proprietary or unpublished data that should be protected by the U.S. Government and should be distributed as approved for public release.

APPENDICES

Supplementary items that are attached to this report are the abstract that was accepted for a poster presentation at the 2013 Military Health Sciences Research Symposium (MHSRS); an article on our research from The Geneva Foundation's *2012 Annual Report*; supplemental figures for the major equipment used in our experiments, i.e., shock tube for generating blast wave injuries, electroretinogram (ERG) machine for recording retinal responses, and operant conditioning chambers for visual discrimination testing (Figures A, B, and C, respectively); and a copy of the poster presented at the 2013 MHSRS.

Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System, in Rats

James C. DeMar, Ph.D., Stephen A. VanAlbert, Miya I. Hill, Robert B. Gharavi, Joseph R. Andrist, Andrea A. Edwards, Cory A. Riccio, and Joseph B. Long

Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of decreasing intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge needed for developing therapies for blast related injuries and to advancing military medicine.

SUPPORT: This work is supported by a USAMRMC/ TATRC Vision Research Program grant award, #: W81XWH-12-2-0082.



DEVELOPING TREATMENTS FOR BLAST-RELATED VISION LOSS

“I can tell you, from my perspective, the signature weapon of this conflict is blast, and blast is a potentially devastating weapon which can burn, can result in amputation of limbs, that can result in loss of eyesight and hearing, that can damage brains and obviously, as we’re all concerned, can lead, because of the context of the conflict for the combatant, to many post-traumatic stress results.”

— LTG Eric Schoomaker, Commander, USAMEDCOM, April 17, 2008¹

Blast injury from detonation of improvised explosive devices (IEDs) has emerged as the most frequent battlefield injury and greatest threat to warfighters in the current operations of Iraq and Afghanistan. Standard penetrating and blunt trauma to the body is the most common injury among survivors, and up to 10% of those afflicted have significant eye injuries². Blast-related eye injuries often occur without any obvious outward signs of trauma, making them difficult to recognize, diagnose, and treat.

A leading cause of vision loss in the warfighter is the result of exposure to blast shock waves and the subsequent non-penetrating traumatic injuries to the eyes and brain visual processing centers³. A

substantial portion of blast-related closed-eye injuries, up to 26%, involve tears, detachments, and hemorrhaging of the retinas.⁴ Based on human clinical studies and recent animal studies, it is of high probability that exposure to even moderate blast waves can lead to neuronal cell death in the retina and brain visual processing centers that is severe enough to cause partial or full blindness.

Permanent loss of vision is a lifelong disability that has a profound impact on the warfighter’s quality of life. In 2012, Dr. James DeMar, a Geneva researcher at the Walter Reed Army Institute of Research (WRAIR), began a research study to address the urgent need for new drug therapies to stop the progression of cell death in the retina and brain as a result of exposure to blast waves. This scenario is especially of concern when eye and brain blast injuries suffered by military personnel are not immediately attended to in the field, continuing the inflammation process and damage to the eye for an extended period of time. Dr. DeMar is specifically interested in studying novel drugs derived from omega-3 polyunsaturated fatty acids,

which are known to be potent anti-inflammatory agents.⁵

The frequency of blast exposure and the resulting blast injuries from recent combat operations have allowed Geneva researchers to draw a more accurate clinical picture of the impact of blasts. The results of blast injury research have and will continue to be instrumental in improving the safety of our warfighters during combat, the quality of life for veterans, and even the well-being of civilians at job sites. This important research conducted by Geneva teams will continue to add to the growing base of knowledge in the treatment and prevention of injuries related to blast exposure.

1. US Department of Defense, Blast Injury Research Program, <https://blastinjuryresearch.amedd.army.mil/index.cfm?f=application.introduction> (Apr. 29, 2011).

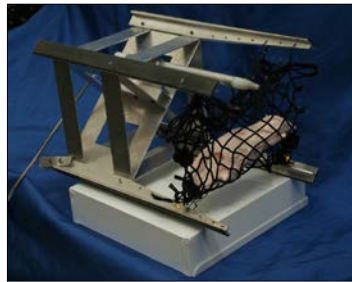
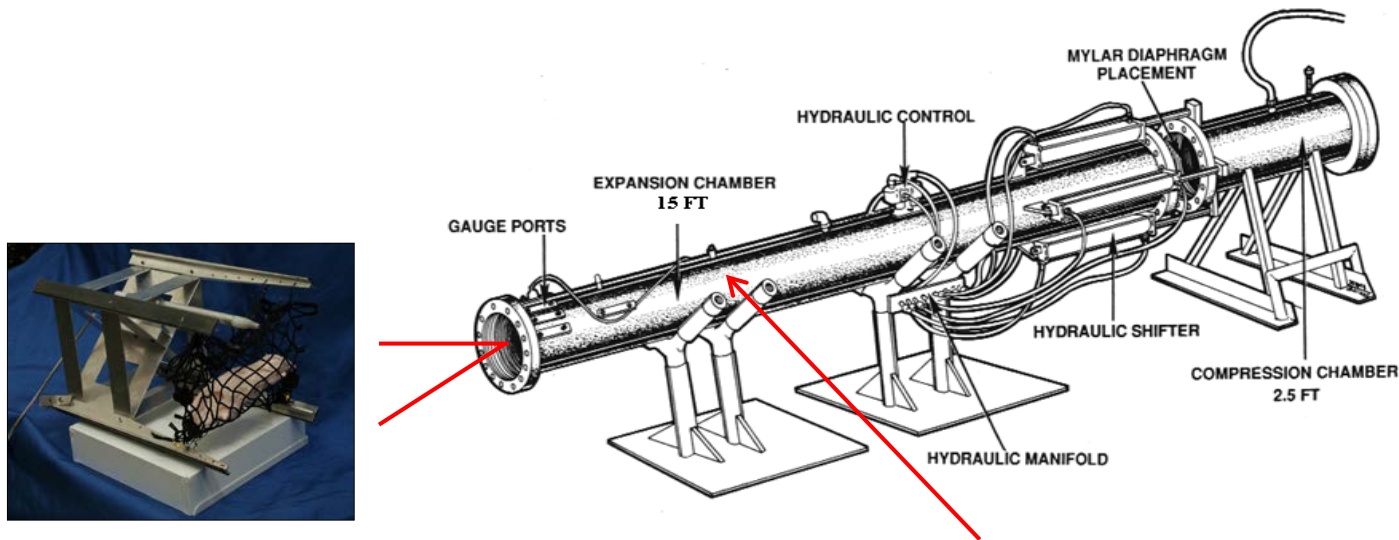
2. Centers for Disease Control and Prevention

3. Capó-Aponte JE, Uroševich TG, Temme LA, Tarbett AK, Navjit K, and Sanghera OD (2012). Visual dysfunctions and symptoms during the subacute stage of blast-induced mild traumatic brain injury. *Military Medicine*, 177, 7:804.

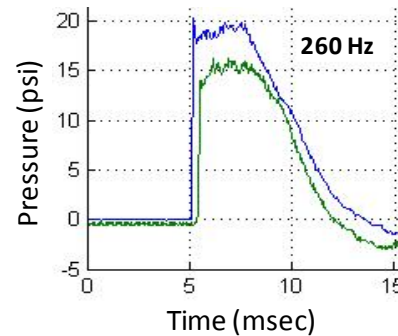
4. Cockerham GC, Rice TA, Hewes EH, Cockerham KP, Lemke S, Wang G, Lin RC, Glynn-Milley C, and Zumhagen L (2011). Closed-eye ocular injuries in the Iraq and Afghanistan wars. *N Engl J Med*. 364(22): 2172-2173.

5. Serhan CN. (2010). Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? *Am. J. Pathol.* 177(4): 1576-1591.

Supplemental Figure A: Shock tube for generating blast wave injuries.



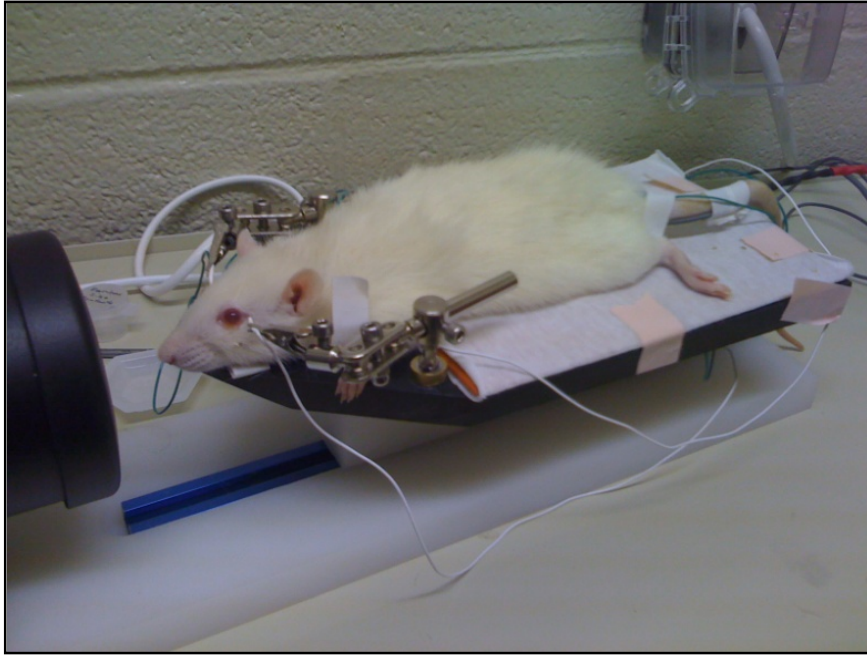
Animal holder (sled with sling)



- Mach 1.34 shock front speed
- 62 μ sec rise time
- 281 mph (126 m/s) blast wind
- Acceleration > 1000 xg

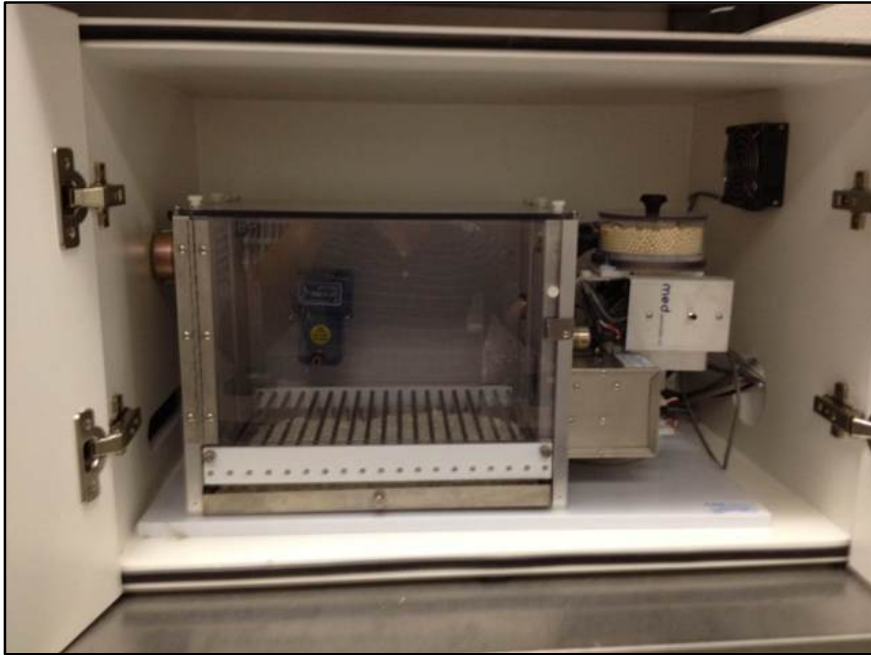
Supplemental Figure A: Schematic diagram of the compressed air driven shock tube for generation of blast wave injuries to the eyes and brains of rats, which is in place at the WRAIR for use by laboratories within the Division of Blast Induced Neurotrauma. During blasting, the shock tube delivers a static pressure of 20 psi at 260 Hz (inset graph) to the position of the animal inside the expansion chamber. The blast wave travels by the rat with a Mach 1.34 shock front speed, 62 μ sec rise time, 6 msec duration, 281 mph (126 m/s) wind speed, and an acceleration g-force of > 1000 g. Also shown is the animal holder, which consists of a metal sled equipped with a nylon mesh sling (mock rat is displayed inside as mounted in a right side on position) that is inserted down into expansion chamber before blast wave exposure.

Supplemental Figure B: Electroretinogram (ERG) machine for recording retinal responses.



Supplemental Figure B: Electroretinogram (ERG) machine for recording retinal signaling responses in rats. Anesthetized rat is shown mounted in a hand held multi-species ERG unit (left panel) as made by Ocuscience Inc. (Kansas City, MO), which is in place at the WRAIR for use by laboratories within the Division of Blast Induced Neurotrauma. Animal is wired with ground (tail), reference (cheeks), and recording (corneas) electrodes; and then face is inserted into a lamp dome (right panel) for exposure to light flashes (0.1 - 25 cd.s/m² intensity; 5 msec duration; 10 sec intervals) as the retina stimulus. Pictures were taken and adapted from sales information openly available on the Ocuscience, Inc. website (<http://www.ocuscience.us/index.html>).

Supplemental Figure C: Operant conditioning chamber for visual discrimination testing.



Supplemental Figure C: Operant conditioning chamber for visual discrimination testing of rats. Wide-open (left panel) and close-up views (right panel) of an operant conditioning test chamber as made by Med Associates, Inc. (St. Albans, VT), which is in place at the WRAIR for use by laboratories within the Division of Blast Induced Neurotrauma. In the wide open view, the internal housing cage is shown, which is equipped with a water bottle and an automatic food pellet dispenser connected to a recessed trough. In the close-up view, two retractable levers (visible slots) are found on either side of the trough that when pressed activate food pellet dispensing. Cue lights are located directly above each lever, as well as trough, and can be used for signaling the animal to press down on them. Chamber has a house light on the ceiling to provide a normal 12/12 h light/dark cycle; and the outer doorway is shut during the test to eliminate outside auditory and visual distractions.



Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System in Rats



^{1,2}James C. DeMar, Ph.D., ^{1,2}Miya I. Hill, ¹Robert B. Gharavi, ¹Joseph R. Andrist, ¹Andrea A. Edwards, ^{1,2}Cory A. Riccio, ^{1,2}Stephen A. VanAlbert, and ¹Joseph B. Long

¹*Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910;*
²*As Contracted Through The Geneva Foundation, Tacoma, WA 98402*



ABSTRACT

Blast injury has emerged as arguably the greatest threat to War fighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of decreasing intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge for developing therapies for blast related injuries and to advancing military medicine.

TIME PERIOD OF STUDY: September 2012 - Present.
SUPPORT: USAMRMC / TATRC Vision Research Program grant, award #: W81XWH-12-2-0082.

INTRODUCTION

□ In recent theaters of operation (OIF and OEF), 80% of the neurotrauma cases in U.S. soldiers resulted from attacks using improvised explosive devices (Warden, 2006).

□ Blast injuries are a leading cause of loss of visual function in War fighters, due to trauma to the eyes and brain visual processing enters (Capó-Aponte, 2012; Cockerham, 2011).

□ Of these afflicted patients, 43% display closed-eye injuries (Cockerham, 2011).

□ Of the ocular injuries, 26% involve the retina, consistent with a blast wave displacement of fragile tissues (Cockerham, 2011).

□ Despite the serious life-long disability loss of vision represents, only a few animal studies have been done to characterize neurotrauma to the visual system resulting from blast wave exposure (Petras, 2007; Hines-Beard, 2012; Mohan, 2013).

References:

Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
Hines-Beard et al., 2012; Exp. Eye Res. 99: 63-70.
Mohan et al., 2013; Invest. Ophthalmol. Vis. Sci. 54(5): 3440-3450.
Petras et al., 1997; Toxicology. 121(1): 41-49.
Warden, 2006; J. Head Trauma Rehabil. 21(5): 398-402.

AIM OF STUDY

Rigorously, characterize in rats exposed to high fidelity simulated blast overpressure waves the cellular, neuronal signaling, behavioral pathology of injuries to the eyes - specifically retina - and brain visual processing centers, as by:

- 1) Electroretinography (ERG).
- 2) Visual discrimination (operant conditioning).
- 3) Histopathology (H&E and silver stains).

MATERIALS AND METHODS

Simulation of Primary Blast Wave Injuries:

□ Adult male Sprague Dawley rats (6 wk-old) are exposed under isoflurane to blast over pressure waves, in a right-side on orientation, using a compressed air driven shock tube.

□ Single air blast of ~20 psi is applied to the rat, via rupture of a Mylar membrane of predetermined thickness.

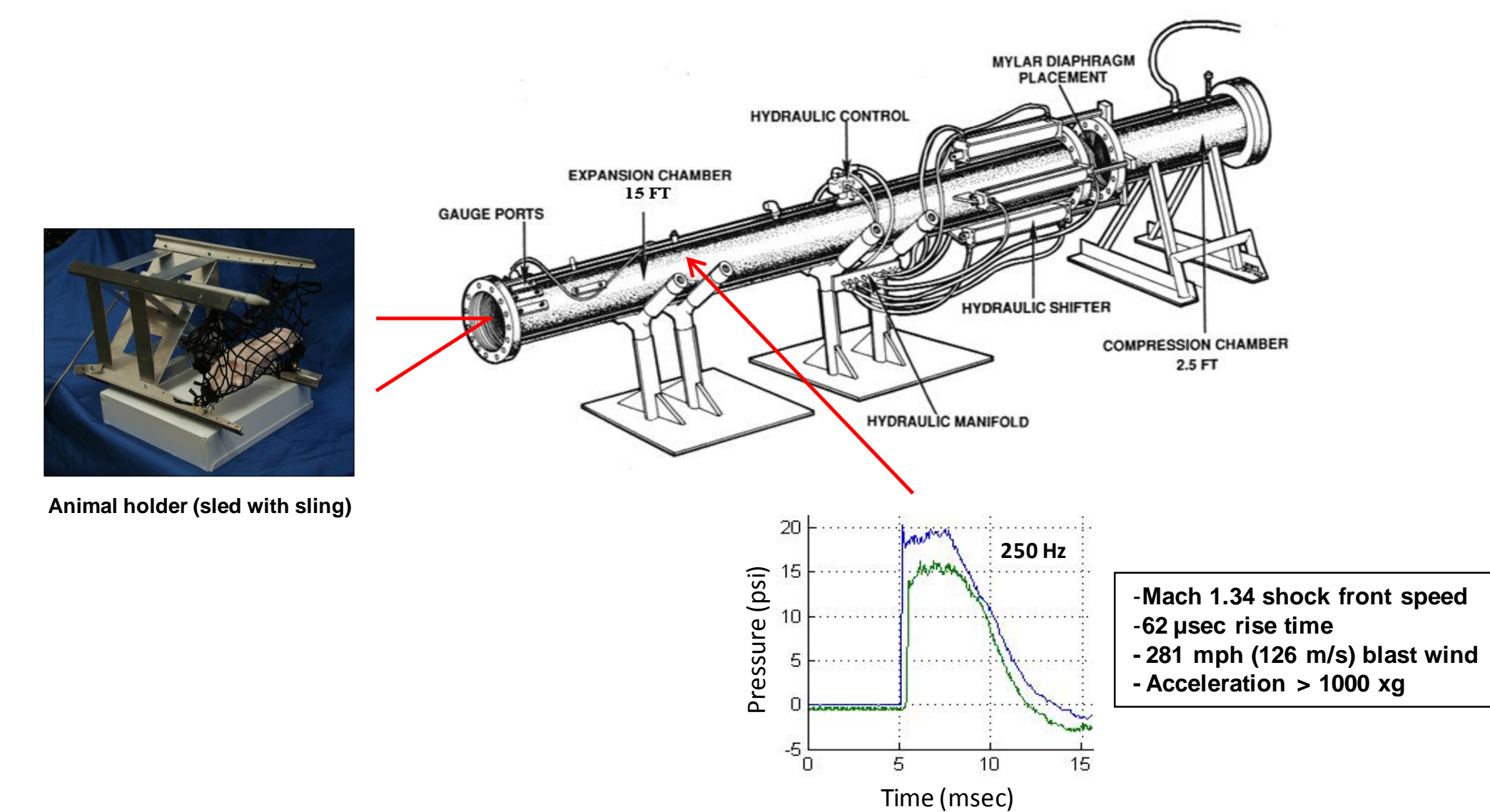


Figure 1. Diagrammatic view of the WRAIR shock tube.

Electroretinography (ERG):

□ Rats are dark adapted for 5 h; and then kept under red lights.

□ Under isoflurane, pupils are drug-dilated; and electrodes put on eyes (recording), cheeks (reference), and tail (ground).

□ Eyes are flashed with light (0.1 - 25 cd.s/m²; 5 msec); and evoked retina potentials are recorded (a- and b- waveforms).

□ Tested at baseline (1 d prior) and 1, 7, and 14 d post-blast.

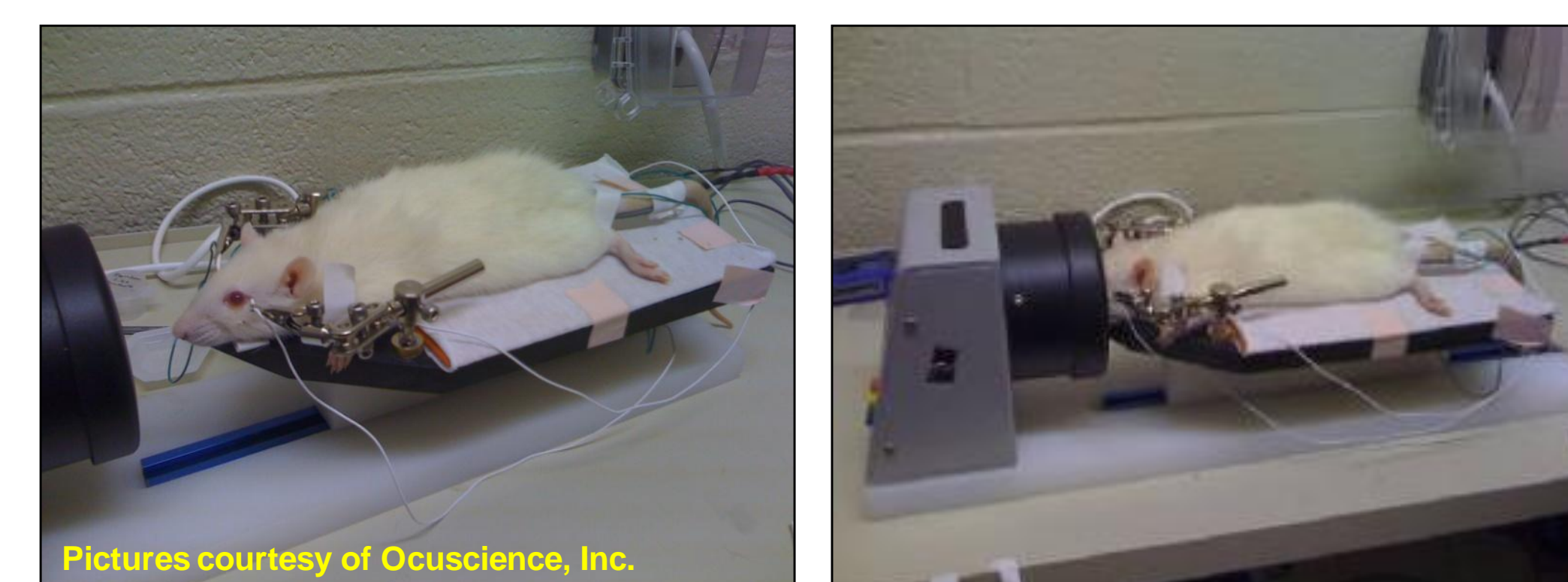


Figure 2. Rat mounted in an ERG instrument (Ocuscience, Inc.).

Visual Discrimination (Operant Conditioning):

□ Rats are trained in operant conditioning boxes over 7 d to press a lever when a cue light shines to gain food rewards.

□ Cue light is then varied in brightness (13 random levels) over next 2 d to challenge visual response, as a baseline prior to blast.

□ Those having a ≥ 60% correct response are continued on.

□ Retested at 2, 5, 7, 12, and 14 d after blast; and data is reported as total, correct, and incorrect lever responses.

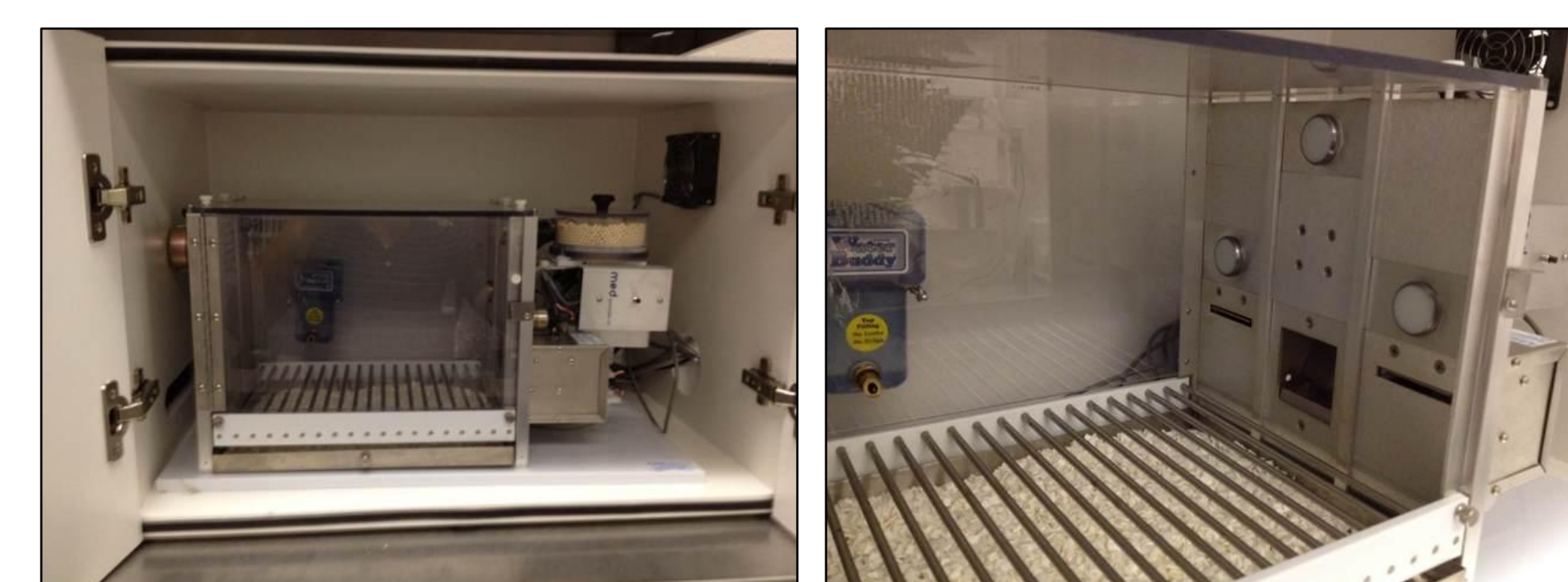


Figure 3. Views of an operant conditioning box (Med Associates, Inc.).

Histopathology (H&E and Silver Stains):

□ Rats are transcardial perfused with paraformaldehyde; and eyes and brains are removed and then post-fixed.

□ Tissue samples are submitted (FD Neurotechnologies, Inc.) for processing into H&E (eyes) and silver (brains) stained slides.

□ Examined under an axial light microscope for damage in retina cell layers and brain visual processing centers; where H&E stains for general cell morphology (pink to purple) and silver for axonal tract degeneration (brown to black).

RESULTS

Electroretinography (ERG):

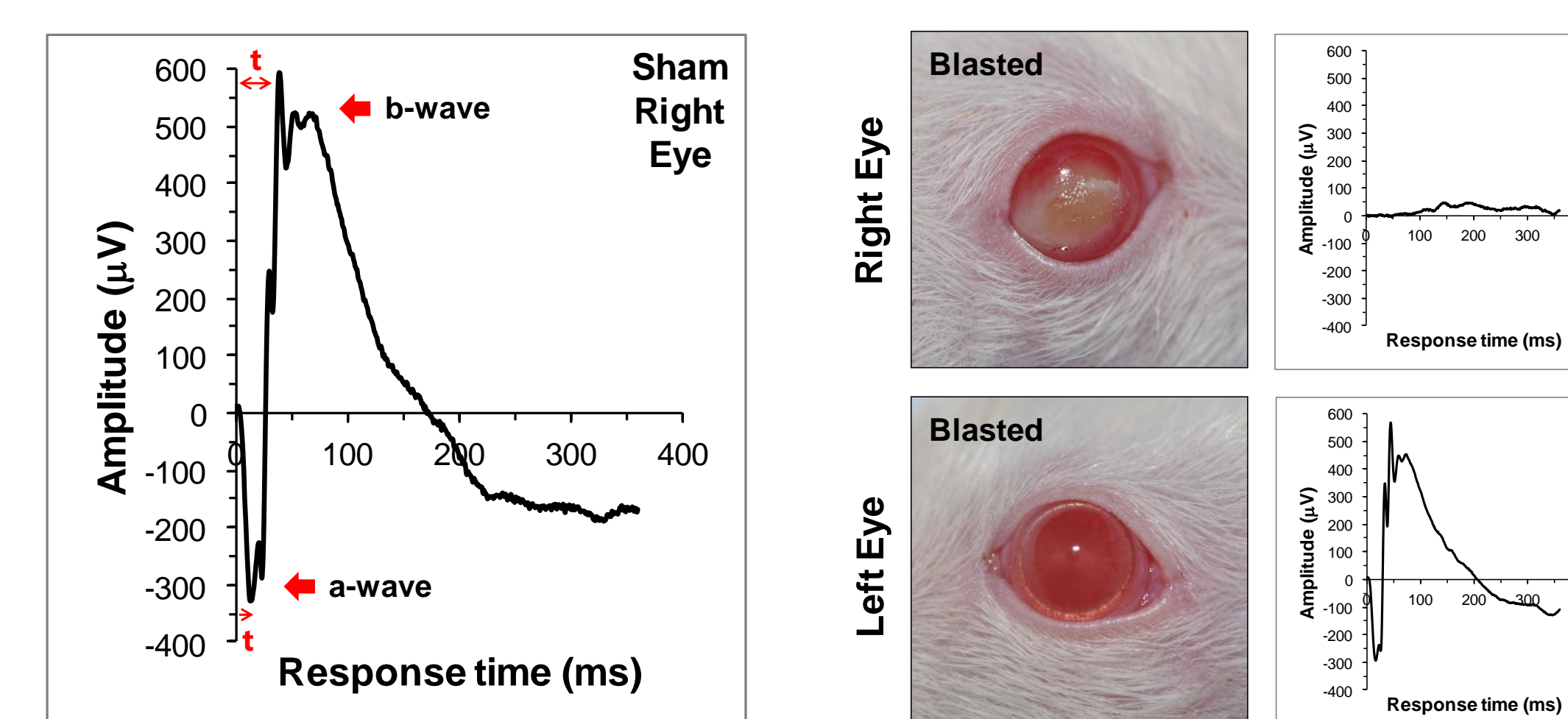


Figure 4. Electroretinogram (ERG) trace showing a- and b-wave responses (1 cd.s/m² flash), from retina photoreceptor and bipolar cell neurons, respectively; t = implicit time. Right and left eyes of a rat at 7 d post-blast, as shown along side their respective ERG traces.

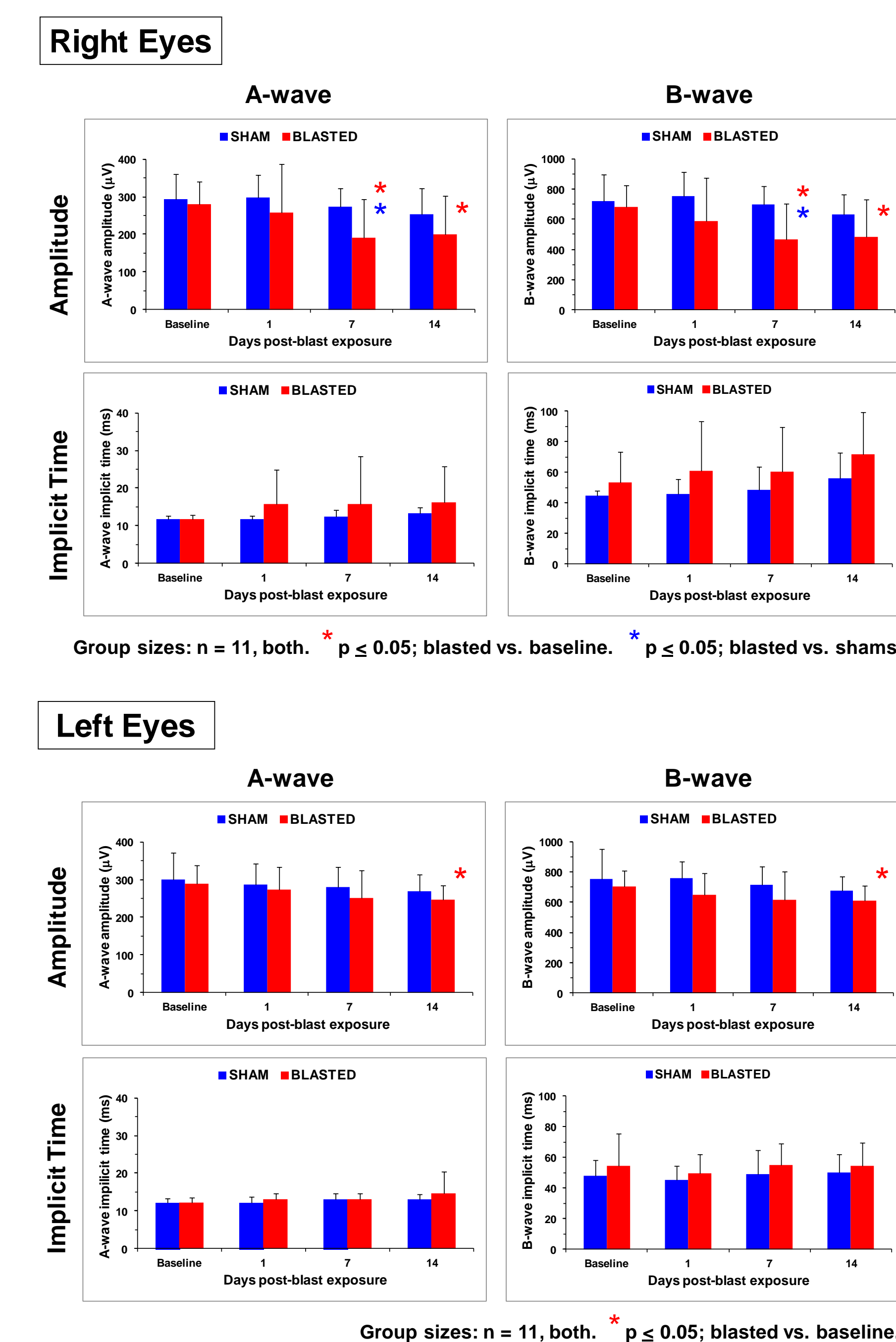
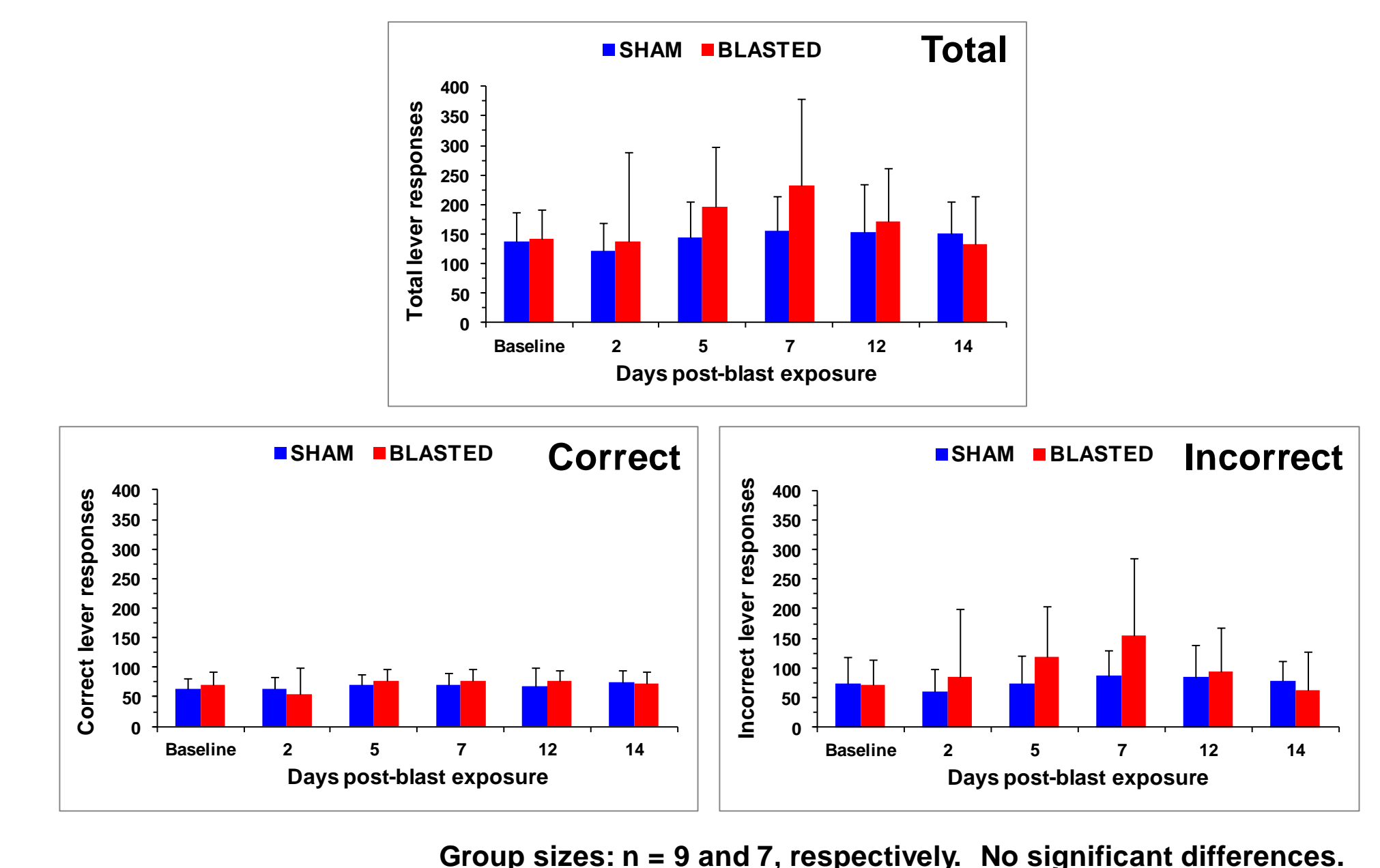


Figure 5. ERG amplitudes and implicit times for a- and b-wave signal responses (3 cd.s/m² flash) of sham and blasted rats (right and left eyes) at baseline and 1, 7, and 14 d after exposure. * p ≤ 0.05, for blasted rats vs. their baseline or shams, as determined by t-test.

Visual Discrimination (Operant Conditioning):



Group sizes: n = 9 and 7, respectively. No significant differences.

Figure 6. Visual discrimination test data for total, correct, and incorrect lever responses to a cue light in attempt to gain food rewards, as taken at baseline and 1, 2, 5, 7, 12, and 14 d post-blast.

Histopathology (H&E and Silver Stains):

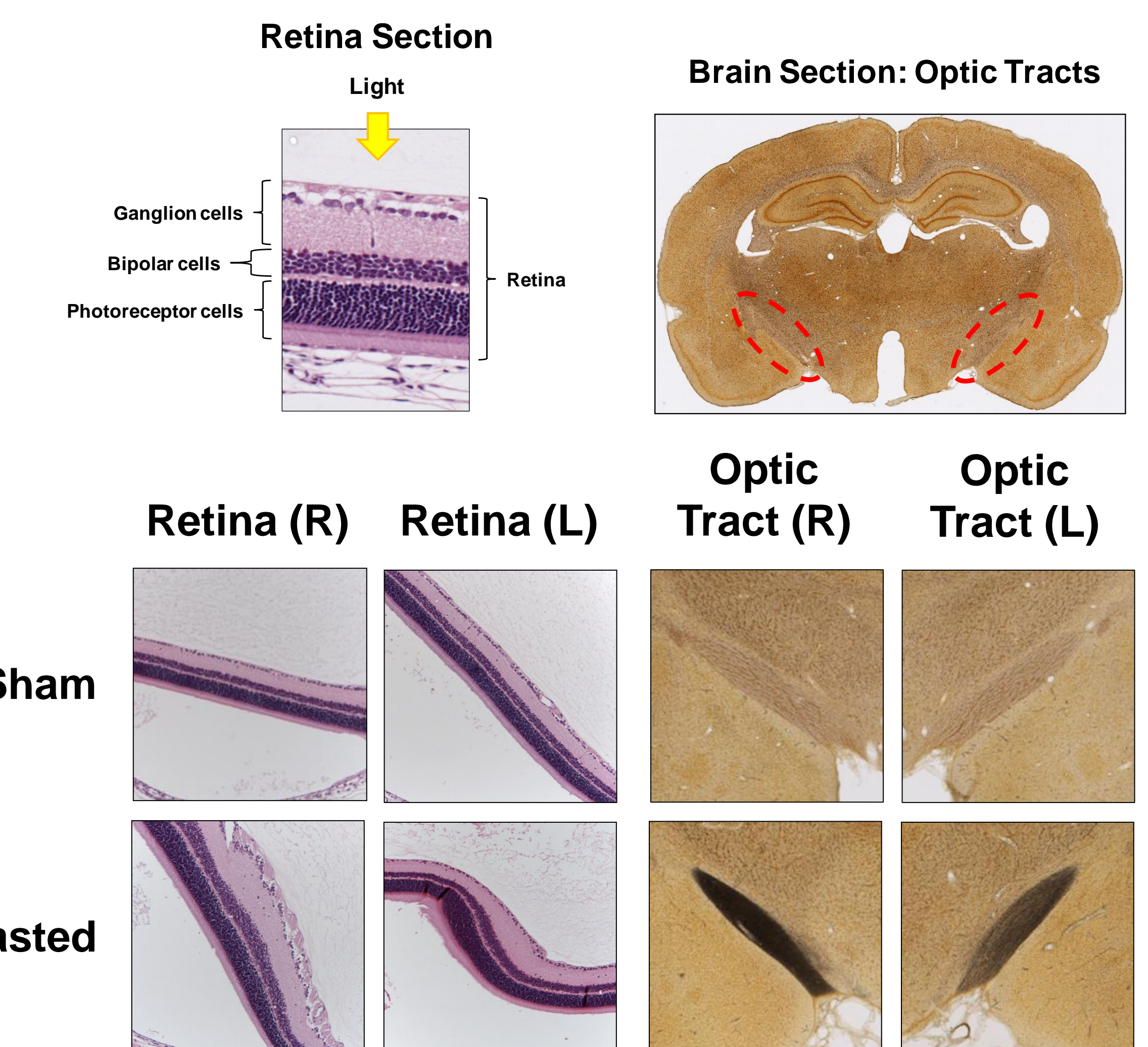


Figure 7. Histopathology of eyes (retina) and brains (optic tract) for representative sham and blasted rats; H&E and silver stains, respectively. Magnifications shown are 4 - 10x. R = right; L = left.

SUMMARY AND CONCLUSIONS

□ Blasted rats had significantly lower ERG exam a- and b-wave amplitudes at 7 and 14 d post-exposure, versus their baseline and sham values, which is a clear sign of retinal dysfunction.

□ Visual discrimination testing showed a trend for the blasted rats to "guess" more for food rewards, over time similar to the ERG results.

□ Histopathology showed cell damage to be markedly present in the blasted rat retinas (swelling) and brain optic tracts (axonal shearing).

DISCLAIMER: Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. Opinions or assertions contained herein are private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals.